RESEARCH ARTICLE

Preliminary genetic linkage maps of Chinese herb *Dendrobium nobile* and *D. moniliforme*

SHANGGUO FENG, HONGYAN ZHAO, JIANGJIE LU, JUNJUN LIU, BO SHEN* and HUIZHONG WANG*

Zhejiang Provincial Key Laboratory for Genetic Improvement and Quality Control of Medicinal Plants, Hangzhou Normal University, Hangzhou 310018, People's Republic of China

Abstract

Dendrobium is an endangered genus in the orchid family with medicinal and horticultural value. Two preliminary genetic linkage maps were constructed using 90 F_1 progeny individuals derived from an interspecific cross between *D. nobile* and *D. moniliforme* (both, $2n = 38$), using random amplified polymorphic DNA (RAPD) and intersimple sequence repeat (ISSR). A total of 286 RAPD loci and 68 ISSR loci were identified and used for genetic linkage analysis. Maps were constructed by double pseudo-testcross mapping strategy using the software Mapmaker/EXP ver. 3.0, and Kosambi map distances were constructed using a LOD score ≥ 4 and a recombination threshold of 0.4. The resulting frame map of *D. nobile* was 1474 cM in total length with 116 loci distributed in 15 linkage groups; and the *D. moniliforme* linkage map had 117 loci placed in 16 linkage groups spanning 1326.5 cM. Both maps showed 76.91% and 73.59% genome coverage for *D. nobile* and *D. moniliforme*, respectively. These primary maps provide an important basis for genetic studies and further medicinal and horticultural traits mapping and marker-assisted selection in *Dendrobium* breeding programmes.

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Introduction

Dendrobium is one of the most prominent genera in the orchid family, with more than 1000 species native to South and Southeast Asia, Australia, New Zealand, and Oceania (Lavarack *et al*. 2000), of which 74 species and two varieties are mainly distributed in the provinces and autonomous regions of the southern Tsinling mountains of China (Tsi *et al*. 1999; Chen and Tsi 2000). In traditional Chinese medicine, *Dendrobium* plants are very valuable medicinal herbs with wide clinical applications. About 40 *Dendrobium* species are used in traditional Chinese medicine in China (Chinese Pharmacopoeia Editorial Committee 2000) because of their medicinal values. *Dendrobium* substances are also been used for many centuries in traditional medicine in other Asian countries (Bulpitt *et al*. 2007).

Dendrobium plants have high horticultural value as its flowers, along with those of *Cattleya*, *Phalaenopsis* and *Oncidium*, are four of the best-known orchids of the world (Wang Y. *et al*. 2007). Since the 18th century, more than 8000 *Dendrobium* hybrids and cultivars have been bred in horticulture through interspecific hybridization for novel flower morphological characteristics (Lavarack *et al*. 2000). However, the ability of selfreproduction in *Dendrobium* is very low and their seedlings grow very slowly under natural conditions, combined with the reasons of their therapeutic effects and high horticultural value, *Dendrobium* stocks are subjected to overexploitation, which lead to the results that most wild *Dendrobium* germplasm resources only survive in certain habitats and are rapidly getting depleted (Wang H. Z. *et al*. 2009).

Genetic linkage maps have been generated for many plants of economic interest using various types of DNA markers, paving the way for further quantitative trait loci (QTL) mapping, positional gene cloning, comparative genomics studies and marker-assisted breeding. Molecular markers used in the development of genetic linkage maps include randomly amplified polymorphic DNA (RAPD) (Williams *et al*. 1990), simple sequence repeat (SSR) (Weber 1990), intersimple sequence repeats (ISSR) (Zietkiewicz *et al*. 1994), amplified fragment length polymorphism (AFLP) (Vos *et al*. 1995), sequence-related amplified polymorphism (SRAP) (Li and Quiros 2001), and others. These DNA markers provide

*For correspondence. E-mail: Bo Shen, bshen65@hzcnc.com; Huizhong Wang, whz62@163.com.
Shangguo Feng, Hongyan Zhao and Jiangjie Lu contributed equally to this work.

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genetic platforms for constructing linkage maps, and some of them were recently applied to *Dendrobium* germplasm identification, genetic diversity and conservation researches (Wang H. Z. *et al*. 2006, 2007, 2009; Yue *et al*. 2006; Gu *et al*. 2007; Ding *et al*. 2008). However, the studies on genetic linkage maps of *Dendrobium* are very few, and there are just two reports on genetic linkage map construction in *Dendrobium* (Xue *et al*. 2010; Lu *et al*. 2012).

In the present study, two widely used Chinese herbs, *D. nobile* and *D. moniliforme*, were selected for construction of experimental populations by cross-pollination and further genetic linkage mapping by applying the double pseudo-testcross strategy based on RAPD and ISSR markers. Our study represents a perfect mapping population comprised of 90 hybrids which would facilitate identification of *Dendrobium* QTLs controlling economically important traits as well as other genetic and genomic studies.

Materials and methods

Plant materials and DNA extraction

The mapping population consisted of 90 F_1 individuals obtained from a cross between the female parent *D. nobile* from Simao (100°96′E, 21°77′N), Yunnan, China; and the male parent *D. moniliforme* from Menghai (100°49′E, 21°95′N), Yunnan, China (figure 1). Wild-stocks of two parents were collected and transplanted in the greenhouse of Hangzhou Normal University (120°19′E, 30°26′N) for further hybridization in 2005. Seeds obtained from this interspecies hybridization were germinated in lightly moistened potting mix at room temperature. Healthy seedlings were transferred to the nursery garden after three months. Finally, a total of 90 seedlings from interspecies hybrids were randomly selected for constructing the molecular genetic linkage map.

Genomic DNA was extracted individually from fresh leaves using the Plant Genomic DNA Extraction kit (Sangon, Shanghai, China). The concentration of genomic DNA samples was determined by NanoDrop^{TM} 1000 (Thermo Fisher Scientific, Wilmington, USA) and checked with 1% agarose gel electrophoresis. At the end, a portion of the DNA was diluted to 20 ng μL^{-1} with elution buffer as templates for PCR amplification.

RAPD analysis

A total set of 500 RAPD primers were synthesized according to nucleotide sequences of primer sets from the University of British Columbia (Vancouver, Canada) and Operon Technologies (Alameda, USA). RAPD amplification was performed using 10 μL of PCR reaction mixture solution containing 1 μL of 10× PCR buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, 20 mM

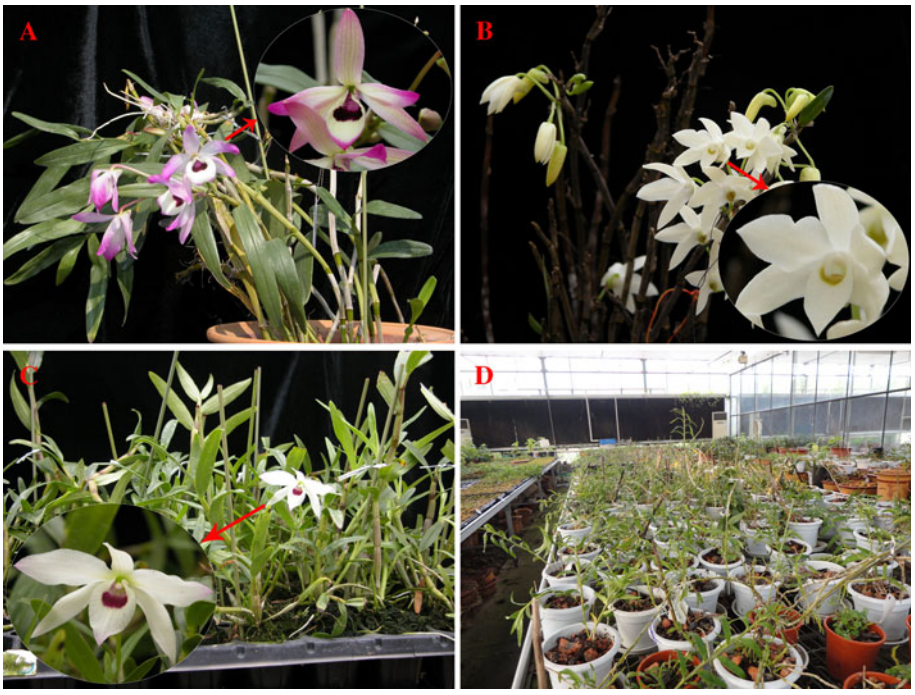

Figure 1. Morphological characteristics of two parents (female parent *D. nobile* and male parent *D. moniliforme*) and their F_1 progeny individuals. (A) *D. nobile*, (B) *D. moniliforme*, (C) and (D) the individuals of the F_1 progeny.

$MgSO_4$, pH 8.8), 1 μL of 1 RAPD primer (10 μM), 0.25 μL of dNTP (10 mM), 20 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (TaKaRa, Kyoto, Japan). Amplifications were carried out in 96-well microplates using a MJ Research PTC-100 thermal cycler (MJ Research, Waltham, USA) programmed for one step of 4 min predenaturation at 94°C, followed by 40 cycles of 30 s denaturation at 94°C, 30 s annealing at 36°C, 60 s extension at 72°C, and a final extension step of 7 min at 72°C. For RAPD marker profiling, PCR products were subjected to electrophoresis on 1.5% agarose gels run at 120 V for 1 h in 0.5× TBE buffer, followed by staining with ethidium bromide. Electrophoresis patterns of the PCR products were recorded digitally using a Gel-Doc 2000 Image Analysis System (Bio-Rad, Philadelphia, USA).

ISSR analysis

For ISSR genotyping, 25 primers were synthesized (Sangon, Shanghai, China) according to nucleotide sequences of primer sets from University of British Columbia (Vancouver, Canada). PCR reactions were carried out as described above. Touch-down PCR program was used for ISSR amplification 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 marker profiling, the PCR products were separated on 6% polyacrylamide gel (acrylamide : bisacrylamide = 19 : 1) and ISSR bands were stained using Silver Sequence DNA Staining kit (Promega, Madison, USA).

Marker scoring segregation test and marker nomenclature

Segregating markers were scored as '1' for presence and '0' for absence for each polymorphic band amplified over parents and sibs. Electrophoresis patterns of DNA markers were documented using the Gel Documentation System (Quantity One Program, BioRad, Philadelphia, USA) and analysed twice. Only repeatable and reliable markers were considered for further analysis. All markers were tested for a fit to the expected 1:1 (presence : absence) ratio using chi-square analysis ($P > 0.05$). The size of each DNA fragment was visually estimated by comparing it with the sizes of DNA molecular standards. The RAPD and ISSR markers were named by primer serial number and the approximate fragment size (bp).

Linkage analysis and genetic map construction

Linkage maps were generated for each parent independently using the pseudo-testcross mapping method (Grattapaglia and Sederoff 1994). Loci heterozygous in one parent and homozygous null in the other were searched in a testcross configuration segregating 1:1. This method is well suited for use in mapping dominant markers such as RAPD, AFLP and ISSR markers (Liu and Ekramoddoullah 2008). Linkage maps were constructed using the mapping program Mapmaker/EXP ver. 3.0 (Lincoln *et al*. 2003). To identify linkage groups, pairwise comparisons and grouping of markers were performed using the 'group' command at a LOD score ≥ 4 and a recombination threshold of 0.4 as described previously. Map distances were computed using the Kosambi mapping function (Kosambi 1944). Linkage maps were drawn using the software MapDraw 2.1 (Liu and Meng 2003). The loci were named according to original primer nomenclature with multiple markers ordered by decreasing molecular weight. The genome length was estimated using the method of moments (Hulbert *et al*. 1988).

Results

Marker analysis and polymorphism loci segregation

A total of 525 primers (500 RAPD and 25 ISSR) were screened on six progeny individuals and two parents to determine their potential for genomic polymorphism identification and reproducibility. Primers revealing DNA fragments polymorphism between parents and segregating in the progeny were selected for subsequent use in the whole mapping population.

For 500 arbitrary RAPD primers, 126 (25.2%) were selected for whole population mapping and they produced 286 polymorphism loci (table 1). The size of the amplified fragments ranged approximately from 150 to 2000 bp. The

Table 1. Markers scored in the 90 progeny of *Dendrobium* using RAPD and ISSR analysis.

	RAPD markers	ISSR markers
Primers screened	500	25
Primers used for mapping	126	16
Testcross markers (1:1) present in *D. nobile*	136	25
Testcross markers (1:1) present in *D. moniliforme*	122	33
Distorted markers	28	10
Total number of markers	286	68
Average number of markers per primer	2.27	4.25

Table 2. Details of the female (*D. nobile*) and male (*D. moniliforme*) genetic maps.

Detail	Map	
	Female (*D. nobile*)	Male (*D. moniliforme*)
Number of linkage groups	15	16
Number of markers	116	117
Total map length (cM)	1474	1326.5
Estimated genome length (cM)	1916.41	1802.53
Average distance between markers (cM)	14.75	14.88

number of markers produced by each RAPD primer ranged from 1 to 4, with an average of 2.27 RAPD loci per primer. Out of 286 loci, a total of 258 (90.21%) were identified as testcross markers ($P > 0.05$), 136 from *D. nobile* and 122 from *D. moniliforme*. In addition, 28 (9.79%) loci showed a distorted 1:1 segregation ($0.01 < P < 0.05$).

For 25 screened ISSR primers, 16 (64%) revealed highly polymorphic patterns and were chosen for the genetic linkage mapping with the whole population. With these 16 ISSR primers, a total of 68 polymorphism loci (table 1) were generated with an average of 4.25 loci per primer, of which 58 (85.30%) were testcross loci (25 *D. nobile* and 33 *D. moniliforme*), and 10 (14.70%) displayed distorted 1:1 segregation.

Genetic linkage map construction

Independent linkage maps were constructed for each parent using the pseudo-testcross mapping strategy (Grattapaglia and Sederoff 1994). A total of 286 RAPD and 68 ISSR polymorphism loci were available for map construction.

For the female parent *D. nobile*, 128 loci (115 RAPD and 13 ISSR loci) were assigned to 15 major linkage groups (DNs, the numbers of *D. nobile* linkage groups) with more than four loci per group (table 2) and four triplets (figure 2). The size of the linkage groups ranged from 35.1 to 191.3 cM, and the number of loci per linkage group varied from 4 to

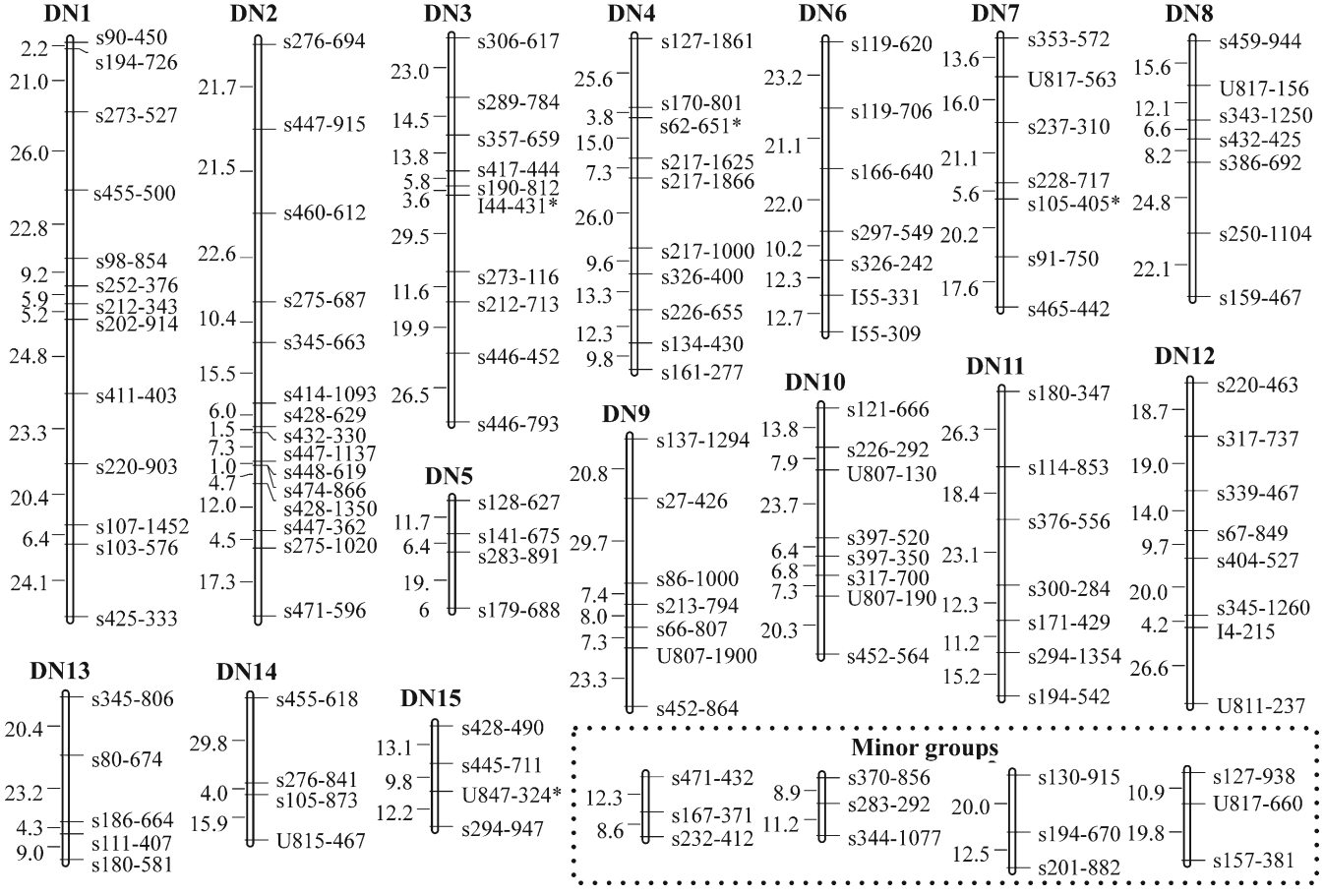

Figure 2. Genetic linkage map of *D. nobile* consisting of RAPD and ISSR markers. Distances in centiMorgans are indicated to the left and marker names to the right of each linkage group. Markers showing distorted segregation are labelled with an asterisk. Four triplets are shown as 'minor groups'.

Table 3. Distribution of markers on *D. nobile* and *D. moniliforme* genetic linkage map.

	D. nobile			*D. moniliforme*		
Linkage group	Total distance (cM)	Number of markers	Average distance (cM)	Total distance (cM)	Number of markers	Average distance (cM)
1	191.3	13	15.94	117.9	17	7.37
2	146	15	10.43	99.6	12	9.05
3	148.2	10	16.47	88.6	12	8.05
4	122.7	10	13.63	92.9	8	13.27
5	37.7	4	12.57	88	6	17.6
6	101.5	7	16.92	127.4	9	15.93
7	94.1	7	15.68	131.8	8	18.83
8	89.4	7	14.9	128.1	6	25.62
9	96.5	7	16.08	74.1	7	12.35
10	86.2	8	12.31	68.1	6	13.62
11	106.5	7	17.75	40.9	4	13.63
12	112.2	8	16.03	39.2	4	13.07
13	56.9	5	14.23	52.6	6	10.52
14	49.7	4	16.57	68.9	4	22.97
15	35.1	4	11.7	53.5	4	17.77
16				55.1	4	18.37

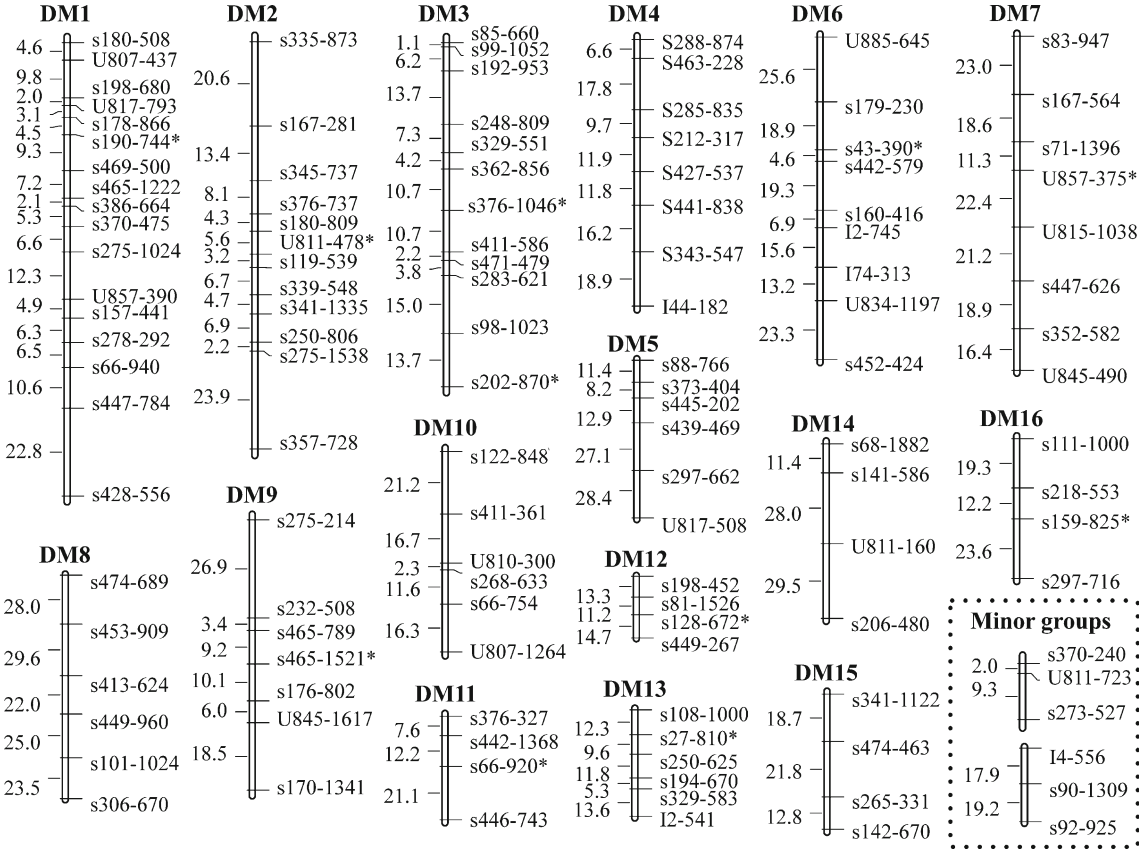

Figure 3. Genetic linkage map of *D. moniliforme* consisting of RAPD and ISSR markers. Distances in centiMorgans are indicated to the left and marker names to the right of each linkage group. Markers showing distorted segregation are labelled with an asterisk. Two triplets are shown as 'minor groups'.

15 (table 3). The average distance between two loci was 14.75 cM. Linkage group DN11 had the largest average distance 17.75 cM, and group DN2 had the smallest average distance 10.43 cM.

For the male parent *D. moniliforme*, 123 loci (102 RAPD loci and 21 ISSR loci) were assigned to 16 major linkage groups (DMs, the numbers of *D. moniliforme* linkage groups) with more than four loci per group (table 2), and two triplets (figure 3). The linkage groups ranged from 39.2 to 131.8 cM in length, and the number of markers per group varied between 4 and 17 (table 3). The average loci spacing was 14.88 cM, ranging from 7.37 cM in linkage group DM1 to 25.62 cM in linkage group DM8.

Expected and observed genome length and map coverage

The 15 maternal linkage groups spanned 1474 cM for *D. nobile*, and the 16 paternal linkage groups spanned 1326.5 cM for *D. moniliforme*. Using the maximum-likelihood method (Chakravarti *et al.* 1991), the total genome sizes were estimated 1916.41 cM and 1802.53 cM for *D. nobile* and *D. moniliforme*, respectively. Consequently, the total linkages constructed here covered 76.91% and 73.59% of the genome for *D. nobile* and *D. moniliforme*, respectively.

Discussion

The parental materials *D. nobile* and *D. moniliforme* selected in this study belong to the section *Dendrobium* and contain the same number of chromosomes ($2n = 38$), but there is a large divergence in terms of phenotype, such as shapes and colours of leaf, flower, and labellum (figure 1). Our previous studies have shown that whether it is based on morphological characters or ISSR molecular markers, these two species were only distantly related (Wang H. Z. *et al.* 2009). Although it is very difficult to obtain a typical family-based population in *Dendrobium* plants, because of their long generation time, out-breeding with high heterozygosity, and inbreeding depression make F_2 or backcross populations rarely available for genetic linkage mapping study. In the present study, a F_1 interspecific hybrid population of these two *Dendrobium* species was created, and 90 seedlings of the F_1 family were used for RAPD and ISSR genotyping and constructed the genetic linkage maps by the double pseudo-testcross mapping strategy. The frame map length in this study was 1474 cM for *D. nobile* with 15 linkage groups and 1326.5 cM for *D. moniliforme* with 16 paternal linkage groups.

Compared to RAPD markers, ISSRs are more efficient but cost more with longer experimental procedures. In our study, each ISSR primer amplified about 4.25 polymorphic fragments on an average, whereas RAPDs detected only 2.27 polymorphic fragments per primer in *Dendrobium*. And segregation test indicated that about 316 (89.27%) of the markers segregated in a strict monogenic Mendelian fashion ($P > 0.05$), including 136 RAPDs and 25 ISSRs from *D. nobile*, 122 RAPDs and 33 ISSRs from *D. moniliforme* in a 1:1 ratio. Most of those markers were informative and mapped on the *Dendrobium* linkages. However, 38 (10.73%) loci segregated in ratios that deviated from Mendelian segregation ratios ($0.01 < P < 0.05$). Segregation distortion might be a common phenomenon in the genetic linkage mapping, and it is worthy of future research. In addition to mapping discrepancies, segregation distortion can also lower the rate of identification of recombinants between loci of interest. In contrast, the inclusion of highly distorted markers ($P < 0.01$) in linkage analysis will lead the untrue distance between the adjacent markers. Therefore, in this study only the markers with the P value > 0.01 were deleted.

The double pseudo-testcross strategy was proposed for mapping populations derived from crossing between two heterozygous parents of outcrossing diploid and polyploid species for which inbred lines cannot be readily generated (Grattapaglia and Sederoff 1994). *Dendrobiums* are outbreeding heterozygous plants and inbreeding depression limits their ability to produce F_2 or backcross populations for genetic linkage mapping. Hence, in this study the double pseudo-testcross mapping strategy and the 90 progenies of the F_1 cross family *D. nobile* × *D. moniliforme* as the mapping population were applied. This method produces independent maps of both parental species and has been successfully applied in other plant species (Grattapaglia and Sederoff 1994; Yin *et al*. 2002; LaRosa *et al*. 2003; Kenis and Keulemans 2005; Lanteri *et al*. 2006; Lowe and Walker 2006; Venkateswarlu *et al*. 2006).

Genetic linkage maps are indirect measures of the number of physical chromosomes and it should be consistent with the haploid chromosome number of the genome when sufficient coverage is achieved. The *D. nobile* map had 15 major linkage groups and the *D. moniliforme* map had 16 major linkage groups, which are close in number to the haploid chromosomal number ($n = 19$). However, there are a few minor linkage groups (figures 2 and 3) in both maps, indicating that the maps were not saturated in both *Dendrobium* species. The discrepancy between the haploid number and the number of linkage groups usually occurred in preliminary genetic linkage maps reported in previous studies on other plant species due to large gaps among DNA markers and low-density marker (Beedanagari *et al*. 2005; Venkateswarlu *et al*. 2006; Cavalcanti and Wilkinson 2007). Additional markers need to be identified for saturation of all 19 linkage groups in *Dendrobium*.

Construction of a genetic linkage map, especially a high-density genetic linkage map requires not only an appropriate mapping population but also a large number of molecular markers. In our lab, the first genetic linkage maps of *D. officinale* and *D. hercoglossum* was reported by using RAPD and SRAP markers (Xue *et al*. 2010). And then, EST-SSR,

SRAP, ISSR and RAPD markers were used to construct the genetic linkage maps of *D. moniliforme* and *D. officinale* (Lu *et al*. 2012). All the preliminary genetic linkage maps of *Dendrobium* species reported were not moderately or highly saturated map needed for the subsequent QTL mapping and marker-assisted selection, and some markers used are not considered to be ideal markers for genetic linkage map construction. Moreover, it is difficult to obtain mapping populations for *Dendrobium* plants, and as nonmodel plants less ideal markers such as SSR and SNP can be used.

In summary, we first constructed some mapping populations of *Dendrobium* species, and proved that these populations were feasible for genetic linkage map construction. Concerning the universal use of genetic linkage maps, codominant markers, including SSRs or SNPs should be the first choice for future mapping efforts, to maximize the marker coverage and distribution in *Dendrobium* genome. To some degree, our genetic linkage mapping results will benefit genomic studies on *Dendrobium* by integrating all the preliminary *Dendrobium* maps into a high-density map.

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