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Development of microsatellite markers for *Dendrobium* orchids

Thanita Boonsrangsom[1], Pradit Pongtongkam[1], Sumon Masuthon[2] and Surin Peyachoknagul[1]*

[1]Department of Genetics, [2]Department of Botany, Faculty of Science, Kasetsart University, Bangkok, 10900 Thailand

**Corresponding author: fscisrp@ku.ac.th*

ABSTRACT

Microsatellite markers for *Dendrobium* orchids were developed using SSR enrichment procedure. Two genomic libraries were constructed from the DNA digested with either *Mse*I or *Taq*I. They were screened for the presence of microsatellite sequences with 6 types of biotinylated oligonucleotide probes ($(CA)_{15}$, $(GA)_{15}$, $(ACC)_{10}$, $(CCT)_{10}$, $(GAT)_{10}$ and $(ATCT)_7$) for DNA digested with *Mse*I and with 4 types of biotinylated oligonucleotide probes ($(CA)_{15}$, $(GA)_{15}$, $(ACC)_{10}$ and $(CCT)_{10}$)) for those digested with *Taq*I. The positive clones were reconfirmed by dot blot hybridization showing 26% and 90% of the respective two groups. The total of 195 positive clones were sequenced, of which 62.8% and 88.8% contained SSR motifs, respectively. Different types of repeat motif were found comprising of GA/TC (50.0%), CCT/AGG (22.8%), 15 types of compound repeat (20.4%), GGGTTTA/TAAACCC (5.6%), CA/TG (0.6%) and GAT/ATC (0.6%). Seventy-three clones were chosen for primer design. Eight primer pairs could be used to amplify the products giving the expected sizes and detect genetic polymorphism in the population with the allele numbers ranging from 4 to 7 (averaged 5.25 alleles per locus), observed heterozygosity (H_o) of 0.0612 to 1.0000 (averaged 0.7398), expected heterozygosity (H_e) of 0.0788 to 0.7323 (averaged 0.5871) and effective number of allele (n_e) of 1.0855 to 3.7355 (averaged 2.7850). The genetic relationships of 49 *Dendrobium* hybrids were analyzed using NTSYS-pc version 2.1m program. The results showed that all *Dendrobium* samples could be identified but they were not clearly separated into distinct clusters. However, the abundance of microsatellite in *Dendrobium* orchids and the high information content of the developed markers will be useful for genetic diversity analysis and cultivar discrimination.

INTRODUCTION

The Orchidaceae is one of the largest and most diverse families of flowering plants, attribute to one tenth of all flowering plant species in the world (Dressler, 1993). Many of which have developed fascinating characteristics to adjust themselves to a wide range of habitats, to attract pollinators and to conserve moisture and nutrients. In several tropical countries, orchids have become a major ornamental export crop, and the demand for their cut flowers has drastically increased over the years. They are exported as flowering-potted plants and cut flowers at 2 billion baht annually. The genus *Dendrobium*, with 900 species or more, is one of the largest genera in Orchidaceae (Bechtel

et al., 1981) and the most popular genus in horticultural industries.

In Thailand, there are more than 1,000 species of orchids, and these come in a bewildering and dazzling range of colours, shapes, habitats, sizes, numbers of flower and leaf (Anupanskun, 1999). Morphological characters have commonly been used to assess their genetic diversity. Molecular methods, however, are more reliable to measure genetic relatedness and evolution. The combination of morphological and molecular approaches could give us a better description of the variation in germplasm, provide a framework for data collection, and reveal relationships between the markers and morphological traits (i.e., linkage to genes) (Carmen *et al.*, 2005). Molecular markers are powerful tools in modern agriculture. They have been used for genome and comparative mapping, phylogeny and population genetics, parental selection and species identification, association studies and QTL analysis.

Microsatellites or simple sequence repeats (SSRs) are the class of repetitive DNA sequences present in all organisms, both eukaryotes (Morgante *et al.*, 2002) and prokaryotes (Gur-Arie *et al.*, 2000). They consist of tandemly arranged repeats of several nucleotides (usually 2-6, in tracts up to 10^2 bp) that are distributed through out the whole genome and are flanked by highly conserved sequences (Chambers and McAvoy, 2000). The length variability of microsatellites is caused by the changes in number of repeat motifs, which can be easily detected by PCR (Weber and May, 1989). Microsatellites are actually the most efficient markers, although limited in use due to the long and laborious step of development. There are several methods to develop microsatellite markers. The traditional method is done by screening the genomic libraries using SSR motifs as probes, but the obtained markers are not very effective. The use of SSR-enriched libraries with selective hybridization of DNA fragments using streptavidin-coated magnetic beads or nylon membrane gives a large number of clones containing microsatellites. The other method for development of SSR marker is based on searching for sequences containing microsatellites deposited in the data bases (EMBL, GenBank) and this method is also used in several plant species, including sugarcane (Pinto *et al.*, 2004), cotton (Qureshi *et al.*, 2004), rice, wheat and barley (Rota *et al.*, 2005). Although the development of microsatellite marker is time-consuming and costly, they have advantages over RFLP, RAPD and AFLP markers, on several aspects, i.e., being PCR-based, multi-allelic, high reproducibility, codominance, fast and easy assaying of genotypes. They have been efficiently applied in genetic diversity studies, population analysis, genotyping and fingerprinting of individuals, cultivar discrimination, genetic mapping and marker-assisted selection in several plant species.

In this paper, we report on the isolation and characterization of microsatellites in *Dendrobium* orchids from genomic libraries enriched in $(CA)_n$, $(GA)_n$, $(ACC)_n$, $(CCT)_n$, $(GAT)_n$ and $(ATCT)_n$ repeats. The newly developed microsatellite markers are subsequently, used for DNA typing of *Dendrobium* orchid genotypes.

MATERIALS AND METHODS

Plant material and DNA extraction

Genomic DNA of the 49 *Dendrobium* orchid samples were isolated from the young leaves, using the protocol described by Dellaporta *et al.* (1983) with some modifications. Approximately 2 g of fresh leaves was ground to fine powder in liquid nitrogen. It was then transferred to a 50 ml centrifuge tube containing 15 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl) and 300 µl of 2-mercaptoethanol. The homogenate was added with 1 ml of 20% SDS and incubated at 65°C for 20 min. Five ml of 5M potassium acetate was added and centrifuged at 20,000 g at 4°C for 20 min. The homogenate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1 (v/v)) and

centrifuged at 3,500 g at room temperature for 10 min. DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After being centrifuged at 3,500 g at room temperature for 10 min, the DNA pellet was washed with 70% ethanol and air-dried. Then, 300 µl of 2M NaCl was added and incubated at 60°C until dissolved. The DNA was ethanol precipitated and the pellet was washed 3 times with 70% ethanol. After removal of RNA by incubation with RNaseA, extraction with chloroform/isoamyl alcohol (24:1 (v/v)), the DNA was ethanol precipitated and resuspened in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Construction of SSR-enriched library and primer designing

SSR-enriched libraries of *Dendrobium* orchids were constructed using the modified procedure of Edwards *et al*. (1996). Briefly, DNA was digested with either *Mse*I or *Taq*I, ligated to *Mse*I adapter (5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5') or *Taq*I adapter (5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCGC-5') and PCR-amplified. Microsatellite sequences were selected using 6 types of biotinylated oligonucleotide probes ($(CA)_{15}$, $(GA)_{15}$, $(ACC)_{10}$, $(CCT)_{10}$, $(GAT)_{10}$ and $(ATCT)_7$) for DNA digested with *Mse*I and with 4 types of biotinylated oligonucleotide probes ($(CA)_{15}$, $(GA)_{15}$, $(ACC)_{10}$ and $(CCT)_{10}$) for those digested with *Taq*I. Bead-based selection was carried out using streptavidin-coated magnetic beads. The selected DNA fragments were amplified by PCR and cloned into pGEM®T plasmid (Promega, USA) following the manufacturer's instructions and the recombinant plasmids were transformed into JM109 supercompetent *E. coli* strain (Promega, USA), which were then plated onto LB agar plates. The plates were incubated overnight at 37°C. The inserts were amplified by colony PCR and reconfirmed by dot blot hybridization with biotinylated oligoprobes (North2South Chemiluminescent Hybridization and Detection Kit, Pierce Biotechnology). The plasmids were extracted from the positive clones using QIAprep®Spin Miniprep Kit (QIAGEN) and sequenced. Primers were designed based on the sequences flanking the microsatellites motifs using the PRIMER 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were designed in 18-25 nucleotide range to yield amplification products of 100-350 bp.

PCR amplification and electrophoresis

PCR amplification was carried out in a final volume of 15 µl containing 25 ng DNA, 1 µl of each primer (forward and reverse primer) (5 pmol/µl), 1 µl dNTP mix (2mM), 1.5 µl 10X PCR buffer, 2.5 mM $MgCl_2$, 0.1 µl *Taq* DNA polymerase (5U/µl) and ultrapure water to the final volume of 15 µl, using PCR program: denaturation at 94°C for 3 min, followed by 35 cycles of 94 °C for 30s, T_a for 60s, 72°C for 40s and final extension at 72° C for 5 min. Amplified products were separated on a 6% denaturing polyacrylamide gel and stained with silver nitrate.

Data analysis

For each microsatellite locus, sizes of the alleles were estimated by comparison with standard size DNA markers and scored across all the 49 *Dendrobium* samples in a binary matrix where 1 represented the presence of a band and 0 for the absence of bands. Genetic parameters, i.e., alleles per locus, effective number of allele (n_e), observed heterozygosity (H_o), and expected heterozygosity ($H_e = 1 - \Sigma p_i^2$, where p_i is the frequency of the i-th allele out of the total number of alleles) were calculated (Hedrick, 1985). The genetic relationships of 49 *dendrobium* hybrids were analyzed using NTSYS-pc version 2.1m program (Rohlf, 1997). The similarity matrix was used for constructing the dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmatic Averages).

RESULTS

Isolation and characterization of microsatellite sequences

Two microsatellite enriched libraries were constructed by digestion of genomic DNA with different restriction enzymes. They were screened for the presence of microsatellite sequences with 6 types of biotinylated oligonucleotide probes for DNA digested with *Mse*I. Due to the self complementarity of oligonucleotide repeats probes between $(ACC)_{10}$ and $(GAT)_{10}$, and $(GA)_{15}$ and $(ATCT)_7$, genomic library enriched for $(CA)_{15}$, $(GA)_{15}$, $(ACC)_{10}$ and $(CCT)_{10}$ microsatellite core motifs were developed by digesting DNA with *Taq*I. The number of SSR containing clones were obtained ranging from 26% in *Mse*I and 90% in *Taq*I libraries (Table 1).

One hundred and ninety-five clones were sequenced from the two libraries. Among them, 122 clones were unsuitable for primer design due to the following reasons: in 89 clones, either the 5′ or the 3′ sequence flanking the SSR motif was too short; 4 clones did not have microsatellite sequence; 29 clones could not be sequenced. The repeat motif in 162 clones sequenced were classified into 51% of dinucleotide repeats, 23% of trinucleotide repeats, 20.4% of compound repeats and 5.6% of short tandem repeats (Table 2). Among these, GA motif containing sequences were obtained as high as 50% but only 0.6% for those of CA motif.

Microsatellite markers

For the clones suitable for primer design, two forward and two reverse primers were designed with the Primer 3 interface program. Four combinations of the primers were tested with the DNA samples. The best primer combination that could amplify product of expected size was chosen. PCR primers were designed for 73 loci. From 73 primer pairs, 8 (11%) produced a clear amplification product within the expected size and could detect polymorphism among the 49 *Dendrobium* hybrids (Table 3, Fig. 1). The other primer pairs did not produce clear-cut PCR products under the PCR conditions used. Other conditions were also tested for these primers, including higher annealing temperatures for primer pairs with several bands (Rafalski *et al.*, 1996) or a touchdown approach for primer pairs with low band resolution (Brown *et al.*, 1996).

Allele sizes were determined using commercial size standards. A total of 42 alleles were produced from 8 markers with an average of 5.25 alleles per locus (Table 4). The highest number of allele (7 alleles) was detected from DR31 primer while the lowest (4 alleles) were obtained from DR26 and DR30 primers with the alleles varying in size from 104 to 230 bp. The expected heterozygosity ranged from 0.0788 to 0.7323 (mean 0.5871) and the observed heterozygosity ranged from 0.0612 to 1.000 with an average of 0.7398. The effective number of alleles (n_e) were found ranging from 1.0855 to 3.7355 (mean 2.7850).

Table 1 Number of clones obtained at different steps.

Item	Genomic libraries digested with	
	*Mse*I	*Taq*I
1. Number of clones from transformation	292	539
2. Number of clones selected by PCR	168	469
3. Number of clones subjected to dot blot hybridization	168	169
4. Positive clones detected by dot blot hybridization	43	152
5. Percentage of positive clones	26	90
6. Number of clones sequenced	43	152
7. Number of clones used for primer development	10	63

Table 2 Distribution of microsatellite classes.

Microsatellite sequences	Genomic libraries digested with	
	*Mse*I	*Taq*I
Dinucleotide repeats		
GA/TC	9	72
CA/TG	-	1
Trinucleotide repeats		
CCT/AGG	1	36
GAT/ATC	1	-
Short tandem repeats		
$(GGGTTTA)_n/(TAAACCC)_n$	9	-
Compound repeats		
$(GT)_n$ and $(GA)_n$	-	2
$(GA)_n$ and $(GT)_n(GA)_n/(CT)_n$ and $(CT)_n(CA)_n$	2	4
$(AGG)_n$ and$(GA)_n$/ $(CT)_n$ and $(CCT)_n$	1	2
$(GT)_n(GA)_n/(GA)_n(GT)_n/(CT)_n(CA)_n$	1	6
$(CA)_n(CT)_n$ and $(CCT)_n(CCA)_n(CCT)_n(CCA)_n$	1	-
$(CCT)_n(CCA)_n(CCT)_n$	-	4
$(GA)_n$ and $(GA)_n(TAGA)_n$		1 -
$(CT)_n(CA)_n(TA)_n$	-	1
$(CCT)_n(CCA)_n$	-	2
$(CCT)_n(CCA)_n(CCT)_n(CA)_n(CT)_n$	-	1
$(GA)_n(GT)_n(GA)_n(GT)_n$	-	1
$(GA)_n(GT)_n(GA)_n$	-	1
$(CT)_nGAA(ATCT)_n(CT)_n$	1	-
$(GGT)_nAAT(GGA)_n$	-	1
$(AAG)_n(AGG)_n$	-	1
Total	27	135

Figure 1 SSR locus DR02 showing 5 alleles amplified from 49 *Dendrobium* orchids. Alleles size ranged from 203 to 224 bp. [M was standard 25 bp DNA ladder, 1-49 were *Dendrobium* samples]

Table 3 Characterization of *Dendrobium* microsatellite markers.

Locus	Primer sequence (5′-3′)	Repeat type	Repeat motif	T_a (°C)
DR02	F: GGACATAAAGAGAGCAGGG	imperfect	$(GAGT)_4GT(GA)_{12}$	50
	R: ATATCCCACCTCCCCCTATC	compound	$(TAGA)_6$	
		repeat		
DR06	F: GGCCAAAGACTCCTAGCTGA	perfect	$(AGG)_{25}$	55-45
	R: CATCTCTCCCCATGCAACTT	repeat		*
DR12	F: AAGGCGGCATCGGCGAATG	imperfect	$(AGG)_{13}GATA(GA)_3$	45
	R: CCTCCCCTTTCTCTCTATTCC	compound	$GT(GA)_2AAA(GA)_4$	
		repeat		
DR26	F:CGATCTTTCACTCTCTCCATCAC	perfect	$(CT)_{18}$	57
	R:ACAAAAGGGGAGAGAGCATGAG	repeat		
DR30	F: CTCTCCATCTTGCCATCCTC	imperfect	$(CT)_3TA(TC)_3$	57
	R: AGCTTGTGGGGAAGGCTGG	repeat	$TA(TC)_3$	
DR31	F: AGAGGGAGAGTGCAAGAG	perfect	$(GA)_{10}$	65-58
	R: CGCTATTTCCATCCCTCC	repeat		*
DR49	F: ACTTGGTGGTCGACGAGGTC	imperfect	$(CCT)_5(CCA)_2(CCT_4$	45
	R: GAGCAAGGGGAGGGATAGAG	compound	$(CA)_3(CT)_6,(CT)_7,$	
		repeat	$(CT)_6ATCTCA(CT)_4$	
DR67	F:CGATAACTATCTATATCCCCCTA	imperfect	$(TC)_3T_3(CT)_2CATTC$	56
	R: AGAGAGCAACAGGAGGAATG	repeat	$A(CT)_6AGA(TC)_3$	

* PCR amplification by touchdown program

Table 4 Allele size range (bp), number of alleles, observed heterozygosity (H_o), expected heterozygosity (H_e) and effective number of allele (n_e) found in *Dendrobium* orchids.

Locus	Allele size range (bp)	No. of alleles	Observed heterozygosity (H_o)	Expected heterozygosity(H_e)	Effective number of allele (n_e)
DR02	203-224	5	0.7045	0.7030	3.3664
DR06	195-208	6	0.8478	0.7323	3.7355
DR12	152-172	5	0.0612	0.0788	1.0855
DR26	169-182	4	0.8125	0.6351	2.7408
DR30	171-183	4	1.0000	0.5794	2.3777
DR31	219-230	7	0.7143	0.6680	3.0120
DR49	190-200	5	0.9697	0.5795	2.3781
DR67	104-129	6	0.8085	0.7209	3.5842
Total		42	5.9185	4.6970	22.2802
Average		5.25	0.7398	0.5871	2.7850

Genetic relationship among Dendrobium hybrids

The data obtained from all 8 primer pairs across 49 *Dendrobium* hybrids were scored and computed to construct a dendrogram (Fig. 2). The result showed that all *Dendrobium* samples could be identified but they were not clearly separated into distinct clusters. The highest similarity coefficient (0.9761) was found in three pairs of *Dendrobium* cultivars, i.e., *D.* Sanan White and *D.* White 5N, *D.* Sonia 17 Red and *D.* Sonia K Wan, and *D.* Burana Green and *D.* Hom 16. Each pair is closely related cultivar of common ancestor and also having similar morphology. The most distant cultivar was *D.* Reha Zehorn, a newly developed cultivar.

DISCUSSION

Two genomic libraries of *Dendrobium* orchids enriched for two dinucleotide, three trinucleotide and one tetranucleotide repeats were developed using SSR enrichment procedure. A total of 162 clones containing microsatellite repeats were identified among 195 clones sequenced. The enrichment procedure was successful, in which 26% and 90% of clones from the *Mse*I or *Taq*I libraries contained microsatellite repeats, respectively. The *Mse*I library had low efficiency because of self complementarity of oligonucleotide probes, so the second *Taq*I library was developed. Several obtained sequences were not suitable for primer design. Microsatellite sequences in most clones are found located too close to the end of insert to accommodate primer design in the flanking region. Some sequences with sufficient length of flanking region were AT rich and unsuitable to use. To overcome this limitation, multiple restriction enzymes were used to digest genomic DNA as also suggested by Hamilton *et al.* (1999). The libraries were enriched for dinucleotide repeats (GA and CA), of the 162 characterized loci, GA repeats were found to be highly obtained (50%) as compared to CA repeats (0.6%). Several research groups also reported on having more GA than CA, including peanut (He *et al.*, 2003), rubber (Roy *et al.*, 2004), and chickpea (Sethy *et al.*, 2006). The most frequently occuring microsatellite dinucleotide repeats in plants are AT with GA and CA as the second and third most frequent (Wang *et al.*, 1994). However, due to the difficulties in screening for (AT) repeats using hybridization techniques we decided to develop microsatellite markers containing (GA) and (CA) repeats.

Only few polymorphic microsatellite markers were obtained from this study (8 polymorphic markers from 73 loci used for primer design). The cause of low polymorphism might come from the use of commercial *Dendrobium* cultivars which had the narrow genetic background. The number of alleles (4-7 alleles per locus) was low as compared to peanut (averaged 8.4 alleles per locus) (Moretzsohn *et al.*, 2004), *Eucalyptus nitens* (averaged 9.5 alleles per locus) (Byrne *et al.*, 1996), and hop (averaged 10.6 alleles per locus) (Stajner *et al.*, 2005) but higher than lychee (averaged 2.06 alleles per locus) (Li *et al.*, 2006), sunflower (averaged 2.67 alleles per locus) (Hvarleva *et al.*, 2007) and bread wheat (averaged 3.5 alleles per locus) (Bryan *et al.*, 1997). The information content (expected heterozygosity, H_e) of the developed markers were compared. The most informative marker was DR06 (H_e 0.7323) and the less informative one was DR12 (H_e 0.0788) with the average of 0.5871. The H_e of some markers were found to be relatively high (H_e 0.322 in lychee (Li *et al.*, 2006), H_e 0.42 in sunflower (Hvarleva *et al.*, 2007) and H_e 0.52 in melon (Ritschel *et al.*, 2004) indicated their potential to discriminate the orchid samples used.

In most loci, the observed heterozygosities were higher than the expected heterozygosities. This may due to the use of *Dendrobium* hybrids which are heterozygous in nature, small samples size and the presence of null alleles. However, this microsatellite markers showed a high potential to be used for cultivar identification, evaluation of

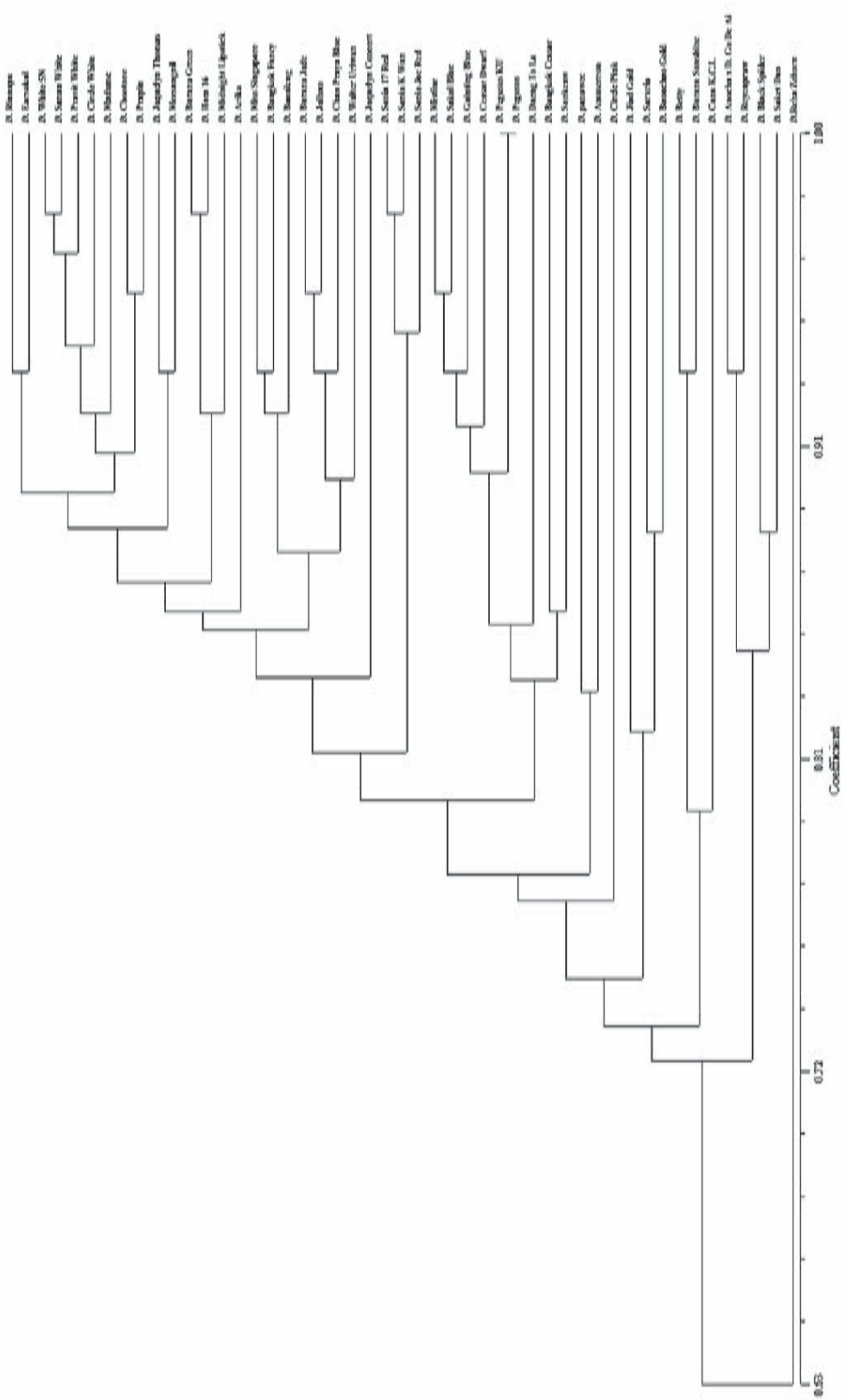

Figure 2 Dendrogram of 49 *Dendrobium* samples calculated from 8 microsatellite marker loci.

the varietal purity in commercial samples, analysis of genetic diversity and also for genome mapping and marker-assisted selection.

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