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Development of Microsatellite Markers for *Vanda* Orchid

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ABSTRACT

The aim of this research was to develop microsatellite (SSR) markers for the *Vanda* orchid from the enriched library using dinucleotide repeats [$(CA)_{15}$ and $(GA)_{15}$] and trinucleotide repeats [$(ACC)_{10}$ and $(CCT)_{10}$] as probes. Positive clones were selected using dot blot hybridization. The results showed that 82.45% of dinucleotide-enriched libraries but only 9.91% of trinucleotide-enriched libraries gave positive signals. After sequencing, 83.12% of the positive clones contained microsatellite repeats. The four most frequently found sequences were the compound repeats of $(GA)_n(GT)_n$ (45.19%), $(GA)_n$ (22.59%), $(CA)_n$ (15.93%) and $(CCT)_n$ (9.26%). Fifty-six pairs of primers were designed and nine primer pairs could amplify the DNA giving the expected PCR product with polymorphism. There was a range from 3 to 9 alleles per locus and the expected heterozygosity (H_e) range was 0.3150-0.7438. Based on the nine loci of these microsatellite markers, the probability of identity (PI) of any two *Vanda* and related orchid cultivars having the same genotype was approximately 1 in 1,000,000. Therefore, these markers could be used for identification of the *Vanda* orchid samples studied. After analyzing the genetic relationships of 33 *Vanda* and related orchid cultivars using NTSYS-pc 2.1m, the result indicated that the *Vanda* and related orchid cultivars could be divided into four groups. The first three groups were strap-leaved *Vanda*, while the fourth one was terete *Vanda,* which was clearly clustered separately from the other *Vanda* groups. This study showed the isolation efficiency of the enrichment procedure, the abundance of microsatellites in *Vanda* orchids and their potential use for the individual identification of *Vanda* and related orchid samples.

Key words: microsatellite markers, SSR markers, orchid, *Vanda*

INTRODUCTION

Microsatellites or simple sequence repeats (SSRs) are short, tandemly repeated DNA sequences spreading throughout the genome. The repetitive unit of microsatellites is generally one to six bases long. Dinucleotide repeats are the most common category of repeat found in the majority of organisms (Jurka and Pethiyagoda, 1995; Tóth *et al*., 2000; Katti *et al*., 2001) and are usually associated with non-coding regions of the genome (Young *et al*., 2000; Temnych *et al*., 2001). Trinucleotide repeats are often found within open reading frames (ORFs) (Young *et al*., 2000) due to their triplet structure. However, in plants, trinucleotide microsatellites are relatively infrequent (Lagercrantz *et al*., 1993; Ma *et al*., 1996), compared to vertebrates and some other organisms. The variable length of microsatellites is caused by changes in the number of repetitive units (Weber and May, 1989).

Microsatellites are polymorphic among

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and within species, multi-allelic and co-dominantly inherited molecular markers, which become the marker of choice in plant genetic studies (Morgante and Olivieri, 1993; Powel *et al.*, 1996). They have been efficiently applied in genetic diversity studies, population analysis, genotyping and fingerprinting of individuals, genetic mapping and marker-assisted selection in many plant species.

There are several ways to obtain microsatellite markers. Screening genomic libraries by hybridizing with SSR probes and sequencing the hybridized positive clones is the traditional but laborious and costly approach. In recent years, several strategies for microsatellite isolation have been developed. The most popular method is SSR-enrichment, which can significantly reduce time and cost (Billotte *et al.*, 2001). Enrichment methods are based on selective hybridization, capturing microsatellite sequences with biotin-labeled probes that are either captured by magnetic beads coated with streptavidin or fixed on nitrate filter (Edwards *et al.*, 1996). The eluted portion after removing non-hybridized DNA was highly enriched for microsatellite isolation, with enrichment efficiency between 50-90% (Butcher *et al.*, 2000).

The orchid genus *Vanda* belongs to the vandaceous family and grows naturally from southern Australia, all the way to India, and north of China. This genus includes more than 40 wild species and numerous hybrids (Anupansakul, 1999). The *Vanda* orchid is considered the most popular and has the highest demand in the orchid family. However, because of various new hybrids produced for the horticultural industry, it is difficult to identify each type of *Vanda* orchid based only on morphological traits. The SSR marker should be a useful tool for genotype identification of these orchid cultivars.

The objectives of this study were to develop microsatellite markers from the *Vanda* orchid using an enrichment method and to identify the selected samples of *Vanda* and related orchid cultivars using these novel microsatellite markers by PCR.

MATERIALS AND METHODS

Plant materials

DNA from *Vanda* Robert's Delight was used for the development of microsatellite libraries. Thirty-three *Vanda* and related orchid samples were used to screen for microsatellite polymorphism (Table 1). Plant materials were provided by Assistant Prof. Chitrapan Piluek, Faculty of Agriculture, Kasetsart University, Bangkok.

DNA extraction

Genomic DNA from 33 *Vanda* and related orchid samples (Table 1) was isolated from the fresh youngest leaves using the method of Dellarporta *et al.* (1983) with some modification. Approximately 3.0 g of young leaves was ground in liquid nitrogen to fine powder and mixed with the extraction buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 0.2% β-mercaptoethanol and 1.3% SDS). The mixture was incubated at 65°C for 20 min, then 5 M potassium acetate was added and the mixture was further incubated at -20°C for 20 min. After centrifugation at 12 000 g for 20 min, the supernatant was transferred and extracted with chloroform:isoamyl alcohol (24:1 v/v). Isopropanol was used to precipitate nucleic acids and the pellets obtained were dissolved in 2 M NaCl. The total DNA was then precipitated using cold absolute ethanol. The precipitates were washed twice with 70% ethanol and the pellets were dissolved in 300 µl of TE buffer. DNA samples were stored at -20°C.

Enriched library construction

Two DNA libraries were constructed: one was enriched for dinucleotide repeats and the other for trinucleotide repeats by the modified method

Table 1 *Vanda* and related orchid samples used in this study.

No.	*Vanda* orchid samples	No.	*Vanda* orchid samples
1.	*Vanda* Robert's Delight	19.	*Vanda* Robert's Delight 'Black'
2.	*Vanda* Gordon Dillon	20.	*Rhynchovanda* Colmari
3.	*Vanda* Golamco's Blue Magic	21.	*Ascocenda* Princess Mikasa Pink
4.	*Vanda* Manuvadee	22.	*Ascocenda* Princess Mikasa Blue
5.	*Vanda* Yellow	23.	*Vanda* Sansai Blue
6.	*Vascostylis* Pine River Blue	24.	*Vanda* Bangkok White
7.	*Vanda denisoniana*	25.	*Vanda* Rasri Gold
8.	*Vanda* Thanuntes x Manuvadee	26.	*Vanda coerulea* 'Red'
9.	*Vanda* Lumpini Red x Manuvadee	27.	*Vanda teres* (terete vanda)
10.	*Vanda* (Rattana x Siriluk) x Manuvadee	28.	*Vanda* Miss Joaquim (terete vanda)
11.	*Vanda* Dares x Kultana	29.	*Vanda* Doctor Anek
12.	*Vanda* Pachara Delight	30.	*Aranda* Christine
13.	*Vanda* Wirat	31.	*Aranda* Woleco (*Vanda* Doctor Anek x *Aranda* Christine)
14.	*Vanda* Robert's Delight '#31'		
15.	*Vanda* Robert's Delight '#56'	32.	*Vanda sanderiana*
16.	*Vanda* Robert's Delight '#358'	33.	*Vanda* Doctor Anek x *Vanda sanderiana*
17.	*Vanda* Robert's Delight '#888'		
18.	*Vanda* Robert's Delight 'Krugao'		

of Yaish and Vega (2003). Briefly, the genomic DNA of *Vanda* Robert's Delight was digested with the restriction enzyme *Mse*I followed by ligation to the *Mse*I adapter. The adapter-ligated DNA fragments were then PCR-amplified with M1 (5′ GACGATGAGTCCTGAG 3′) primer. Both dinucleotide and trinucleotide repeats were sequentially enriched by hybridization to biotinylated $(CA)_{15}$, $(GA)_{15}$ and $(CCT)_{10}$, $(ACC)_{10}$ probes, and were captured using streptavidin-coated magnetic beads. The captured DNA was eluted and then PCR-amplified with M1 primer. The PCR products were cloned into pGEM-T vector (Promega) and transformed into the bacterial competent cells. The cells were plated on LB-agar containing 100 μg/ml ampicillin, 40 μg/ml IPTG and 40 μg/ml X-Gal.

Colony screening

White colonies were selected and the inserted DNAs were amplified with M13 forward and M13 reverse primers. The positive clones were confirmed by dot blot hybridization (Pierce Biotechnology) using oligonucleotide probes.

Sequencing of inserts and primers design

All positive clones were cultured overnight and their plasmids were extracted using QIA prep® Spin Miniprep (QIAGEN) for sequencing. The obtained sequences were classified according to the type and the presence or absence of microsatellite repeats. PCR primers were designed from the flanking regions of the repeat sequences. All primers were tested and optimized against 14 samples of *Vanda* orchid cultivars.

Amplification of microsatellite loci

The selected PCR primers were used for DNA typing of 33 cultivars of the *Vanda* orchid. The total volume of each PCR reaction was 15 μl, consisting of 50 ng of genomic DNA, 1xPCR buffer, 5 pmol of each primer, 200 μM dNTP and 0.5 U *Taq* DNA polymerase. The PCR program

was: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, annealing temperature (depending on the primer) for 1 min, and 72°C for 30 s, and final extension of 72°C for 5 min and hold at 4°C. PCR products were then electrophoresed in 6% denaturing polyacrylamide gel for 3.5-4 hours at 300 voltage and visualized by silver staining.

Data analysis

The observed heterozygosity (H_o) of each microsatellite locus was calculated as the number of heterozygotes at a locus divided by the total number of individuals surveyed. Expected heterozygosity (H_e) was calculated with Equation 1:

$$H_e = 1 - \sum_{i=1}^{k} p_i^2 \quad (1)$$

where p_i is the frequency of the i^{th} allele of the locus.

Effective number of allele (n_e) was calculated with Equation 2:

$$n_e = 1 / \sum_{i=1}^{k} p_i^2 \quad (2)$$

Probability of identity (PI) was obtained using Equation 3:

$$PI = \sum_{i=1}^{k} (p_i^2)^2 + \sum_{i=1}^{k} \sum_{j=i+1}^{k} (2p_i p_j)^2 \quad (3)$$

where p_j is the frequency of the $(i+1)^{th}$ allele of the locus.

The genetic relationship of the studied *Vanda* and related orchid samples was also analyzed by the NTSYS-pc 2.1m program (Rohlf, 2000).

RESULTS AND DISCUSSION

Development of microsatellite markers

The microsatellite-enriched libraries were constructed from the genomic DNA of *Vanda* Robert's Delight using the enrichment procedure with some modification. A total of 429 clones were collected from the dinucleotide repeats library [$(CA)_{15}$ and $(GA)_{15}$], and checked for the presence of DNA insert using PCR, resulting in 342 positive clones (79.72%). These positive clones were further tested for the presence of microsatellite repeats using dot blot hybridization and 282 positive clones (82.45%) were obtained (Table 2). The high outcome percentage indicated that the development of microsatellite markers using the enrichment procedure was more efficient than using the un-enriched library screening method that only produced less than 1% of positive clones (Tang *et al*., 2003).

For the trinucleotide repeats library [$(ACC)_{10}$ and $(CCT)_{10}$], 323 clones from 340 clones contained inserted DNA (95%). After confirming with dot blot hybridization, only 32 (9.91%) clones were positive for microsatellite repeats (Table 2). The low number of trinucleotide repeats obtained here could have been due to the fact that most of trinucleotide repeats in the *Vanda* orchid are short and imperfect so trinucleotide

Table 2 Number of clones from each stage in marker development.

Successive stages in marker development	Library	
	Dinucleotide repeats	Trinucleotide repeats
1. Transformed colonies	429	340
2. Inserted clones checked by PCR	342	323
3. Positive clones confirmed by dot blot hybridization	282	32
4. Clones sequenced	278	30
5. Primers designed	56	0

probes may be easily removed during the highly stringent washing phase in the hybridization process.

The 308 positive clones from the two DNA libraries were cultured overnight and their plasmids were extracted for sequencing. The obtained sequences were classified into four types according to the presence or absence of microsatellite repeats. The first type was a perfect repeat with both flanking sequences. This type was composed of 121 dinucleotide repeat sequences and 2 trinucleotide repeat sequences. Specific PCR primers could be designed from these sequences. The second type was a sequence having a microsatellite repeat close to the cloning site (114 dinucleotide repeat sequences and 22 trinucleotide repeat sequences), which could not be used to design primers. Perhaps the recognition site of the enzyme *Mse*I is 5′TTAA 3′ and the flanking region of the microsatellite sequence was extremely AT-rich, so the flanking region of the microsatellite was cut out. The third type was sequences without a microsatellite repeat (9 from the dinucleotide repeat libraries and 3 from the trinucleotide repeat libraries) and the last type was unreadable and short sequences (34 from the dinucleotide repeat libraries and 3 from the trinucleotide repeat libraries).

The microsatellite sequences were classified as dinucleotide, trinucleotide and compound repeats (Table 3). The $(GA)_n$ sequence was the most abundant in the dinucleotide repeats (22.59%) followed by the $(CA)_n$ sequence (15.93%). This agreed with earlier reports suggesting an abundance of dinucleotide repeats in plants such as peanut (He *et al*., 2003) and avocado (Ashworth *et al*., 2004). Furthermore, Wang *et al*. (1994) surveyed short tandem repeats in many plants and reported that $(AT)_n$ was the most abundant repeat followed by those of $(GA)_n$ and $(CA)_n$. In this study, the $(AT)_n$ probe was not used due to secondary configuration resulting from pairing between A and T nucleotides so this type of repeat was not found except in the compound repeats. For trinucleotide repeats, the $(CCT)_n$ sequence was the most abundant (9.26%) followed by the $(ACC)_n$ sequence (0.74%), which had not been reported before, while $(GA)_n(GT)_n$ was the

Table 3 Types of microsatellite repeats.

Type of microsatellite repeats	Number of clones	Percent
Dinucleotide repeats		
$(CA)_n$	43	15.93
$(GA)_n$	61	22.59
Trinucleotide repeats		
$(CCT)_n$	25	9.26
$(ACC)_n$	2	0.74
Compound repeats		
$(CA)_n(TA)_n$	3	1.11
$(GA)_n(CA)_n$	2	0.74
$(GA)_n(GT)_n$	122	45.19
$(GA)_n(TA)_n$	1	0.37
$(CA)_n(CT)_n(GT)_n$	1	0.37
$(CA)_n(TA)_n(CA)_n$	1	0.37
$(GA)_n(GT)_n(TA)_n$	4	1.48
$(GA)_n(GT)_n(CT)_n$	4	1.48
$(GA)_n(GT)_n(GA)_n$	1	0.37
Total	270	100

most abundant sequence in the compound repeats (45.19%).

Fifty-six PCR primer pairs were designed from the dinucleotide repeat sequences but no PCR primer pairs could be designed from the trinucleotide repeat sequences. This was possibly due to the non-unique flanking regions of the designated sequence, e.g., being extremely AT-rich or due to self-complementarity. Subsequently, the PCR reactions were optimized and seven primer pairs (C32, C106, C208, C225, C268, C359 and C364 (Table 4)) were selected as they could amplify the fragments with the right size and they had polymorphic band patterns. Furthermore, two additional primer pairs developed from the *Mokara* orchid (a related orchid genus) were also used as markers to clearly identify these orchid cultivars. The sequences of all DNA clones used for primer designation were submitted to the GenBank with the accession numbers shown in Table 4.

DNA typing for identification of *Vanda* orchid cultivars

All nine primer pairs were used to examine microsatellite polymorphism in 33 cultivars of the *Vanda* and related orchid samples. The results showed that the number of alleles ranged from 3 to 9 with an average of 6 alleles per locus (Table 5). The primers C106, C208, C225, C268, C359, MOK 26 and MOK29 could amplify DNA from all samples of the *Vanda* and related orchids and had polymorphic DNA fragments (alleles) due to the deletion or insertion of microsatellite DNA caused by slippage or unequal crossing over (Levinson and Gutman, 1987).

The primer C32 could not amplify DNA from some samples of the *Vanda* orchid (null alleles). This may have been due to the deletion of all microsatellite regions in that position (Callen *et al*., 1993) or the mutation of the flanking region (Lehman *et al*., 1996). Moreover, the primers C32 and C364 could not amplify DNA from *Vanda*

Table 4 Characteristics of nine microsatellite markers.

No.	Marker	Primer sequences (5′-3′)	Repeat	T_a (°C)	GenBank Accession number
1.	C32	F: AAT GGA CCT TCT TTG CAT TAC R: ATT ACC GTT CAT TTC TGG TGC	$(GT)_{40}(GA)_{27}$	46	FJ539050
2.	C106	F: AAG TCT AGC TTT TGG TTG AGG R: ATC GAT GGT TTG TTC TTC TAG C	$(TA)_5(GT)_{45}(GA)_{25}$	44	FJ539051
3.	C208	F: TCA TTG ATG TTG GGA GCC TAA R: CTT GCC CTC TAT CTT TCT CTT	$(TA)_3(GT)_{42}(GA)_{10}$	50	FJ539052
4.	C225	F: AGA ACT AGA TGA CTT CAA AAC G R: GAA CTC AGA AAA ATT ACC GCG	$(GT)_6(GA)_{24}$	47	FJ539053
5.	C268	F: TGG AAA TGC ATG TTG CCC GA R: ACT GAG TGA CCT TGG AAG AC	$(GT)_{17}(GA)_{39}$	46	FJ539054
6.	C359	F: CTT TGA GTA ATG TCT CTC AGT G R: CCC TCA CGC ACT CTC TAC C	$(GA)_{15}(GT)_{15}$	45	FJ539055
7.	C364	F: AGC ATT ATA GAA CTA GAT GAC R: GAA TAC TCA AGC TAT GCA TC	$(GT)_{21}$	44	FJ539058
8.	MOK26	F: AGA ATG AGG GAG GTA TAG GG R: TGC CTT GGA TGT GCG TTC G	$(CCT)_{17}$	52	FJ539056
9.	MOK29	F: TTC AGC GTT TCC ATG TCG AAG R: AGT AAA GCC GCC ATC TTG G	$(GA)_{13}$	52	FJ539057

Table 5 Number of alleles, allele size range (bp), observed heterozygosity (H_o), expected heterozygosity (H_e), effective number of alleles (n_e) and probability of identity (PI) found in *Vanda* samples.

No.	Primer	Number of alleles	Size range (bp)	Observed heterozygosity (H_o)	Expected heterozygosity (H_e)	Effective number of alleles (n_e)	Probability of identity (PI)	Combined PI
1	C32	3	193-204	0.4828	0.5684	2.3168	0.2502	0.2502
2	C106	4	193-205	0.6364	0.5394	2.1710	0.3163	0.0791
3	C208	8	153-190	0.7273	0.6012	2.5077	0.2070	0.0164
4	C225	9	206-263	0.3030	0.4839	1.9377	0.2783	4.5590×10^{-3}
5	C268	5	187-212	0.4242	0.5495	2.2197	0.2430	1.1078×10^{-3}
6	C359	6	152-167	0.8788	0.7394	3.8369	0.1114	1.2341×10^{-4}
7	C364	6	296-316	0.9355	0.7438	3.9031	0.1105	1.3637×10^{-5}
8	MOK26	8	212-272	0.8484	0.6877	3.2023	0.1460	1.9910×10^{-6}
9	MOK29	5	155-175	0.3030	0.3150	1.4598	0.4843	9.6425×10^{-7}
Average		6		0.6155	0.5809	2.6172	0.2386	

teres and *Vanda* Miss Joaquim, a terete *Vanda*. This may have been due to the differences in the genetic background between terete *Vanda* and strap-leaved *Vanda*.

The observed heterozygosity (H_o) for individual loci ranged from 0.3030 to 0.9355, with an average of 0.6155. The expected heterozygosity (H_e) ranged from 0.3150 to 0.7438, with an average of 0.5809 (Table 5). These values were comparable to those found in other orchid microsatellite markers, such as *Gymnadenia conopsea* (Gustafsson and Thorân, 2001; Campbell *et al*., 2002) and *Serapias vomeracea* (Pellegrino *et al*., 2001). The effective number of alleles (n_e) ranged from 1.4598 to 3.9031, with an average of 2.6172. Some markers had a low value of n_e , for example, primer MOK29 had five alleles but its heterozygosity and n_e were the lowest because some alleles were found in almost all samples (common allele) while some alleles were rare (rare allele). Therefore, these kinds of primers were not appropriate to use in the identification of the *Vanda* orchid because of low polymorphism.

The probability of identity, PI was the lowest (0.1105) in primer C364. Thus, the possibility of finding any two samples having the same genotype at this locus is about 1 in 9 samples. The more markers used, the lower the value of probability of identity that will be obtained. Considering the nine loci of these microsatellite markers, the probability of identity (PI) of any two samples having the same genotype was 9.6425×10^{-7} or approximately 1 in 1,000,000. Therefore, these markers could be used with a very high level of confidence in the identification of *Vanda* and related orchid samples.

Genetic relationship of *Vanda* orchids used

The genetic relationship of 33 *Vanda* and related orchid samples was analyzed by the NTSYS-pc 2.1m program. The dendrogram (Figure 1) based on shared alleles clearly differentiated all of the samples tested. With a

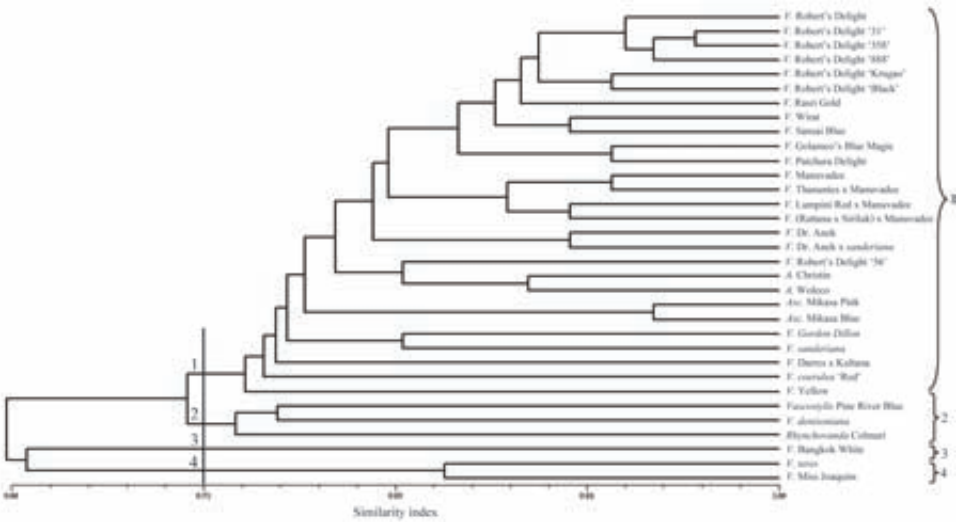

Figure 1 Genetic relationship of 33 *Vanda* and related orchid cultivars analyzed by the NTSYS-pc 2.1m program.

similarity index value of 0.75, the *Vanda* and related orchid samples could be divided into four groups. The first three groups were strap-leaved *Vanda*. The first group was the main group composed of 25 *Vanda* and 2 *Ascocenda* samples. The second and third groups were distinctively separated from the main group, indicating that *Vascostylis* Pine River Blue, *Vanda denisoniana, Rhynchovanda* Colmari and *Vanda* Bangkok White had little genetic relationship with the other *Vanda* samples. The fourth group was terete *Vanda* composed of 2 *Vanda* samples (*Vanda teres* and *Vanda* Miss Joaquim). This group was clearly clustered out from the other *Vanda* and related orchid cultivars because terete *Vanda* has little genetic relationship with the strap-leaved *Vanda* and has been re-classified in the genus *Papilionanthe* (Sin *et al*., 2002). This result confirmed the grouping based on morphological or botanical characteristics, showing the distinctive difference between strap-leaved *Vanda* and terete leaved *Vanda*, and at the same time further distinguished the closely related cultivars of the *Vanda* orchid.

CONCLUSION

This study reported on the development of microsatellite markers from an enriched-DNA library of the *Vanda* orchid. From the dinucleotide repeat library, 82.45% of clones were positive from dot blot hybridization and from the trinucleotide repeat library, 9.91% of clones were positive. After sequencing, 83.12% of the positive clones contained microsatellite sequences, and the $(GA)_n(GT)_n$ repeat was the most abundant microsatellite sequence in the *Vanda* orchid (45.19%) followed by the $(GA)_n$ repeat (22.59%) and the $(CA)_n$ repeat (15.93%). Fifty-six primer pairs were designed and nine primer pairs (including two primer pairs developed from the *Mokara* orchid) were used. After testing the microsatellite polymorphism in 33 samples of the *Vanda* orchid, the numbers of alleles ranged from 3 to 9, with an average of 6 alleles per locus. The observed heterozygosity (H_o) ranged from 0.3030 to 0.9355, with an average of 0.6155 and the expected heterozygosity (H_e) ranged from 0.3150 to 0.7438, with an average of 0.5809. Considering nine loci of these microsatellite markers, the

probability of identity (PI) was 9.6425×10^{-7}, which means that the probability of any two samples having the same genotype was approximately 1 in 1,000,000. PI was the lowest in primer C364, indicating that this was the best primer for identification of the *Vanda* and related orchid samples. Based on the genetic relationships of 33 *Vanda* and related orchid samples analyzed by NTSYS-pc 2.1m, the *Vanda* and related orchid samples could be divided into four groups. The first three groups were strap-leaved *Vanda*. The fourth group was terete *Vanda,* which was clearly clustered out from the other *Vanda* and related orchid samples.

The results clearly indicated that these microsatellite markers have high potential in cultivar identification, the evaluation of cultivar purity in commercial samples and other applications.

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LITERATURE CITED

Anupansakul, M. 1999. **Orchids**. Kaset Book Publication Press, Nonthaburi.

Ashworth, V.E.T.M., M.C. Kobayashi, M. de la Cruz and M.T. Clegg. 2004. Microsatellite markers in avocado (*Persea americana* Mill.): development of dinucleotide and trinucleotide markers. **Scientia Hort.** 101: 225-267.

Billotte, N., A.M. Risterucci, E. Barcelos, J.L. Noyer, P. Amblard and F.C. Baurens. 2001. Development, characterization, and across-taxa utility of oil palm (*Elaeis guineensis* Jacq.) microsatellite markers. **Genome** 44: 413-425.

Butcher, P.A., S. Decroocq, Y. Gray and G.F. Moran. 2000. Developmental, inheritance and across-species amplification of microsatellite markers from *Acacia mangium*. **Theor. Appl. Genet.** 101: 1282-1290.

Callen, D.F., A.D. Thompson, Y. Shen, H.A. Phillips, R.I. Richards, J.C. Mulley and G.R. Sutherland. 1993. Incidence and origin of null allele in the $(AC)_n$ microsatellite markers. **Am. J. Hum. Genet.** 52: 922-927.

Campbell, V.V., G. Rowe, T.J.C. Beebee and M.J. Hutchings. 2002. Isolation and characterization of microsatellite primers for the fragrant orchid *Gymnadenia conopsea* (L.) R. Brown (Orchidaceae). **Cons. Genet.** 3: 209-210.

Dellaporta, S.L., J. Wood and J.B. Hicks. 1983. A plant DNA minipreparation: version II. **Plant Mol. Biol. Rept.** 1: 19-21.

Edwards, K.J., J.H.A. Barker, A. Daly, C. Jones and A. Karp. 1996. Microsatellite libraries enriched for several microsatellite sequences in plants. **Biotechniques** 20: 758-760.

Gustafsson, S. and P.A. Thorân. 2001. Microsatellite loci in *Gymnadenia conopsea*, the fragrant orchid. **Mol. Ecol. Notes** 1: 81-82.

He, G., R. Meng, M. Newman, G. Gao, R.N. Pittman and C.S. Prakash. 2003. Microsatellite as DNA markers in cultivated peanut (*Arachis hypogaea* L.). **PMC Plant Biol.** 3: 1-6.

Jurka, J. and C. Pethiyagoda. 1995. Simple repetitive DNA sequences from primates: compilation and analysis. **J. Mol. Evol.** 40: 120–126.

Katti, M.V., P.K. Rangekar and V.S. Gupta. 2001. Differential distribution of simple sequence repeats in eukaryotic genome sequences. **Mol. Biol. Evol.** 18: 1161–1167.

Lagercrantz, U., H. Ellegren and L. Anderson. 1993. The abundance of various polymorphic microsatellite motifs differs between plants

and vertebrates. **Nucl. Acids Res.** 21: 1111–1115.

Lehman, T., W.A. Hawley and F.H. Collins. 1996. An evolution of evolutionary constraints on microsatellite loci using null alleles. **Genetics** 144: 1155-1163.

Levinson, G. and G.A. Gutman. 1987. High frequencies of short frameshifts in poly-CA/TG tamdem repeats borne by bacteriophage M13 in *Echerichia coli* K-12. **Nucl. Acids Res.** 15: 5323-5338.

Ma, Z.Q., M. Roder and M.E. Sorrells. 1996. Frequencies and sequence characteristics of di-, tri- and tetra-nucleotide microsatellites in wheat. **Genome** 39: 123-130.

Morgante, M. and A.M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. **Plant J.** 3: 175–182.

Pellegrino, G., D. Cafasso, A. Widmer, M. Solina, A. Musacchio and S. Cozzolino. 2001. Isolation and characterization of microsatellite loci from orchid *Serapias vomeracea* (Orchidaceae) and cross-priming to other *Serapias* species. **Mol. Ecol. Notes** 1: 279-280.

Powell, W., G.C. Machray and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. **Trends Plant Sci.** 1: 215-222.

Rohlf, F.J. 2000. NTSYS-pc: **Numerical Taxonomy and Multivariate Analysis System**. Setanket, New York.

Sin, H. C., Y. T. Wing and J. Arditti. 2002. **Biology of *Vanda* Miss Joaquim.** 1st ed. National University of Singapore Press, Singapore.

Tang, B., Y.H. Huang, L. Lin, X.X. Hu, J.D. Feng, P. Yao, L. Zhang and N. Li. 2003. Isolation and characterization of 70 novel microsatellite markers from ostrich (*Struthio camelus*) genome. **Genome** 46: 833-840.

Temnych, S., G. DeClerck, A. Lukashova, L. Lipovich, S. Cartinhour and S. McCouch. 2001. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. **Genome Res.** 11: 1441-1452.

TÛth, G., Z. G·sp·ri and J. Jurka. 2000. Microsatellites in different eukaryotic genomes: survey and analysis. **Genome Res.** 10: 967-981.

Wang, Z., J.L. Weber, G. Zhong and S.D. Tanksley. 1994. Survey of plant short tandem DNA repeats. **Theor. Appl. Genet.** 88: 1-6.

Weber, J.L. and P.E. May. 1989. Abundant class of human DNA polymorphisms which can be typed using polymerase chain reaction. **Am. J. Hum. Genet.** 44: 388-396.

Yaish, M.W.F. and M. Pârez de la Vega. 2003. Isolation of $(GA)_n$ microsatellite sequences and description of a predicted MADS-box sequence isolated from common bean (*Phaseolus vulgaris* L.). **Genet. Mol. Biol.** 3: 337-342.

Young, E.T., J.S. Sloan and K.van Riper. 2000. Trinucleotide repeats are clustered in regulatory genes in *Saccharomyces cerevisiae*. **Genetics** 154: 1053-1068.