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Full Length Research Paper

Molecular characterization and phylogenetic relationships among and within species of *Phalaenopsis* (Epidendroideae: Orchidaceae) based on RAPD analysis

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Random amplified polymorphic DNA (RAPD) analysis for 20 species of *Phalaenopsis* was conducted to determine their genetic distances and relationships. Among 20 different primers used for RAPD analysis, 10 primers showed polymorphism, and according to the primer type, 26 to 54 DNA fragments were amplified. A total of 414 polymorphic fragments were generated by 10 primers and used for correlation group analysis. The highest value of Similarity index was 0.28 between *Ph. violacea malaysia* and *Ph. violacea witte*. The dendrogram resulting from UPGMA (Unweighted Pair Group Method using Arithmetic average) hierarchical cluster analysis separated the original species into three groups: The first group had five species of *Ph. violacea blue*, *Ph. belina*, *Ph. violacea malaysia*, *Ph. violacea witte,* and *Ph. gigantea*; the second group included *Ph. lamelligera, Ph. amabilis, Ph. parishii, Ph. labbi nepal, Ph. speciosa, Ph. lobbi yellow, Ph. venosa, Ph. hieroglyphica,* and *Ph. maculata*; the third group consisted of *Ph. minho princess, Ph. leopard prince, Ph. mannii, Ph. modesta, Ph. cornucervi* and *Ph. pantherina*. RAPD markers can thus be successfully applied in this economically important group of orchids for the study of molecular characterization and relationships. The data acquired from this study could be used for identification and classification of other orchid genera and oriental *Phalaenopsis.*

Key words: *Phalaenopsis*, Random Amplified Polymorphic DNA (RAPD), classification, molecular characterization.

INTRODUCTION

Orchidaceae is one of the most highly developed monocotyledonous families. They are known for their large number of species (775 genera consisting of 19,500 species), great variations in floral morphology (three sepals, two lateral petals and one labellum), pollinator relationships and diversity of their ecological habitat (terrestrial or epiphytic; vines with rhizomes, corms, root tubers or occasionally with mycoparasitic fungi) (Arditti, 1992; Judd et al., 1999). The family, which includes *Cattleya, Dendrobium, Epidendrum, Paphiopedilum,*

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Phalaenopsis, Yangda, Brassica, Cymbidium, Laelia, Miltonia and *Oncidium*, is economically important because of the orna-mental value (Judd et al., 1999).

The genus *Phalaenopsis* belongs to the family Orchidaceae, subfamily Epidendroideae, tribe Vandeae and subtribe Aeridinae (Dressler, 1993; Criley, 2008). Its natural distribution is from India, through South-East Asia to the Philippines, New Guinea and Australia (Sweet, 1980). *Phalaenopsis* is the most popular epiphytic monopodial orchid (Batchelor, 1983), which is grown for commercial production of cut flowers and potted plants. In the United States, Japan and many European countries, not only the domestic production but also the import of this orchid has increased rapidly. Malaysia is one of the twelve mega biodiversity countries blessed as the home of 800 species, covering 120 genera of orchids (Teo, 1995). Orchid industry has become an important role under the support of National Agricultural Policy (NAP, 1992-2010). The orchid industry in Malaysia has grown tremendously and the main destination of Malaysia fresh cut flowers orchids export was Singapore at 52%, followed by Japan 22%, Australia 17%, with the total export value of RM 11,360,387 in year 2001 (FAMA, 2004). By means of automatic production and enterprising management, Malaysia has successfully developed its *Phalaenopsis* industry.

Members of the genus are epiphytes on trees, generally in the shade and in the proximity of water (Davis, 1949). Some species may also grow as lithophytes (Comber, 1972; Fighetti, 2004). *Phalaenopsis* plants have short stems usually with three to six leaves. The leaf blades are usually longer than broad (Batchelor, 1982; Christence, 2001; Griesbach, 2002), either mottled with purplish undersurfaces or light to dark green in color and are usually fleshy and leathery. The flowers are resupinate, vary in size and are usually fleshy and waxy. The flowers are pink, purple, white, brown, yellow or red with the lip or labellum of the flower having the most complex and unique structure (Batchelor, 1982; Stubbings, 2006).

Horticulturally, *Phalaenopsis* is a very important genus, where the wild species are often used as parent plants for breeding purposes. The genus is very popular (Batchelor, 1983) and the demand for them has been phenomenal (Pertwee, 1998), since they first appeared as a 'contender' in the orchid industry in 1992.

The taxonomy of *Phalaenopsis* is confusing. One of the main problems in its systematics is that in the past, different workers have classified in different ways the numerous species in *Phalaenopsis sensu lato* including species from *Doritis* Lindl., *Kingidium* Hunt, *Paraphalaenopsis* Hawkes and *Phalaenopsis* Blume *sensu stricto.*

Due to the splitting or lumping of these four genera, the number of species in the genus *Phalaenopsis* has varied. Rolfe (1886) recognized 34 species in the genus, Sweet (1980) 46, Bose and Bhattacharjee (1980) approximately 70, Shim (1982) 36 species, Teo (1985) 70, and Christenson (2001), 102 taxa of which 62 are species.

As can be seen, opinions often differ and there is a need for another study of the genus using more robust characters such as RAPD markers, other than morphological characters. RAPD analysis is a fingerprinting method using short, random, oligonucleotide primers to search for variation in the entire genomic DNA (Williams et al., 1990; Chang et al., 2000; Chang, 2008) and has been widely employed in evaluating genetic distances in many diverse plant genera, e.g., *Acacia* (Casiva et al., 2002), *Cicer* (Sudupak et al., 2002), *Cupressus* (Rushforth et al., 2003), *Linum* (Fu et al., 2002) and *Rhizophora* (Lakshmi et al., 2002).

In RAPD analysis, sources of DNA polymorphisms may include base changes within the priming site sequence, deletions in the priming site, insertions that render priming sites too distant to support amplifications, and deletions or insertions that change the size of a DNA fragment without preventing its amplification (Williams et al., 1990). The RAPD technique has several advantages such as the ease and rapidity of analysis, a relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (Williams et al., 1990).

In orchids, most of the work that utilised RAPD analysis has concentrated on population studies of one to a few species of orchids, for example, *Goodyera procera* (Wong and Sun, 1999; Chen and Chen, 2003; Arus, 2000), *Eulophia sinensis, Zeuxine gracilis, Z. strateumatica* (Sun and Wong, 2001), *Changnienia amoena, Paphiopedilum malipoense* and *Ph. rnicranthum* (Li et al., 2002). There have been very few studies on the usage of RAPD to address the relationships of taxa at the species level. Lim et al. (1999) worked on the genus *Yanda* and Benner et al. (1995) detected high levels of inter- and intra-specific polymorphisms in the genus *Cattleya* Lindl using RAPD markers. Some of the earlier RAPD studies with *Phalaenopsis* include that of Fu et al. (1997) who worked on 16 species of *Phalaenopsis* and concluded, based on RAPD data and karyotype analyses by previous workers, that *Phalaenopsis* is probably polyphyletic. Been et al. (2002) separated 33 species of

Phalaenopsis into eight groups, of which only two were congruent with those of the morphology-based classification of Sweet (1980).

An adjunct method to the morphological and physiological techniques used for classification is a test based on isozyme expression, which has been introduced to fingerprint species and ornamental cultivars of various species (Deloose, 1979; Chapparro et al., 1987; Obara-Okeyo and Kako, 1998). However, DNA based methods have many advantages compared to the isozyme technique. DNA content is independent of environmental conditions and the DNA sequence is identical in all plant tissues or tissue stages (Erlich et al., 1991). The development of highly reliable and discriminatory methods for identifying species and cultivars has become increasingly more important to plant breeders and members of the nursery industry who need sensitive and accurate tools to differentiate and identify cultivars for the purpose of plant patent protection. A number of molecular techniques, which include gene mapping and gene sequencing, are available for generating and analyzing molecular data (Judd et al., 1999). Molecular data has played an essential role in determining the genetic relationship among many plants, and has led to new genetic classifications that often conflict with traditional taxonomy (Jobst et al., 1998). In *Phalaenopsis* species, RAPD (Fu et al., 1997; Feng et al., 2003; Goh et al., 2005) and RFLP and inheritance patterns of chloroplast DNA in intergenic hybrids of *Phalaenopsis and Doritis* (Chang et al., 2000; Ching-chun et al., 2006) and somaclonal variation (Chen et al., 1998) in *Phalaenopsis* studies have been reported. Phylogeny can also be used to understand the evolutionary process, which leads to the development of hypothesis concerning subjects, such as morphological adaptation, physiological changes or biogeography (Lazaro and Aguinagalde, 1996; Dubouzet et al., 1998; Kim et al., 2005).

The objectives of this study were to present a comprehensive phylogenetic reconstruction of *Phalaenopsis* based on RAPD molecular data and to evaluate the results with respect to phylogenetic relationships and classifications.

MATERIALS AND METHODS

To determine the relationships of the genus *Phalaenopsis,* the experiment was conducted between 2006 - 2008 in which eighteen species and two hybrids of *Phalaenopsis* were analyzed. For most species, more than two accessions were included for DNA extractions (Table 1) because RAPD analyses of multiple accessions of a given taxon would increase the chances that intraspecific variation be detected if present (Goh et al., 2005). Independent extraction of DNA from each accession was carried out to determine reproducibility of the results in repeated analyses using the same accessions.

Plant materials

All plants' materials were obtained from *in vitro* seed culture established at the Agro-technology laboratory, University Putra, Malaysia. A list of the species sampled is provided (Table 1 and Figure 1). Healthy leaves were collected from each plant. The leaf samples were first washed with tap water and surface sterilized with 10% (v/v) Clorox® solution (Chlorox (Malaysia) Sdn. Bhd., Malaysia) for 5 min. Following this, they were rinsed three times with distilled water and blotted dry with paper towels. They were then kept in plastic containers or wrapped with aluminum foil and stored at - 80°C or used immediately for DNA extraction according to Goh et al. (2005).

Total genomic DNA extraction

Total DNA was extracted from young leaves collected, following the procedure described by Doyle (1991), using the cetyl trimethyl ammonium bromide (CTAB) method with minor modifications by Goh et al. (2005) and adapted to Orchid as follows: 1.5 g young leaves were ground in liquid nitrogen to fine powder and extracted with CTAB hot extraction buffer [100 mM Tris-HCl, pH = 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1 % (w/v) PVP (polyvinyl pyrrolidone) and 2% (v/v) β-mercaptoethanol]. The mixture was incubated at 60°C for 1 h, followed by two extractions with chloroform/isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids, and the pellet obtained was dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH = 8.0 and 1 mM EDTA, pH = 8.0). Co-precipitated RNA was removed by digestion with RNase A. Remaining impurities were extracted with processed phenol and chloroform. Total DNA was precipitated using sodium acetate and cold ethanol. The precipitate was washed twice with 10 mM ammonium acetate in 76% ethanol, and the DNA pellets were air-dried and subsequently dissolved in 50 µl TE buffer. The purified total DNA was quantified by gel electrophoresis, and its quality verified by spectrophotometry (Sambrook et al., 1989). DNA samples were stored at 4°C.

PCR amplification and product electrophoresis

A pre-screening of one set of primers (from MWG Biotech, Germany and Geneset, France) was performed using one of the cultivars from Malaysia. Ten decamer oligonucleotides were used for polymerase chain reaction (PCR) amplification following the procedures of Williams et al. (1990) with some modifications (Table 2). To optimize the PCR amplification condition, experiments were carried out with varying concentrations of $MgCl_2$ (2, 2.5 and 3 mM) and DNA template (10, 20 and 40 ng/µl). The dNTPs (0.2, 0.3 and 0.4 mM) and primers (1 µM) were used as optimized

Table 1. Comparison of origin, lineage, morphological and ecological traits for 20 species of Phalaenopsis.

Species	Origin	Lineage	Main characteristics		
			Ecology	Stem	Roots
Ph. minho princess	Unknown[a]	Phal Sun Prince x Phal Ta Lin Freeds	Epiphytic and Lithophytic	long	Flexuous, glabrous.
Ph. leopard prince	Unknown[a]	Dtps Sun Prince x Phal Ho's French Fantasia	Epiphytic and Lithophytic	long	Flexuous, glabrous.
PH. hieroglyphica	Philippines[a]	Unknown	Epiphytic	short	Fleshy, not very flexible.
Ph. maculata	Malaysia, Borneo	Unknown	Epiphytic and Lithophytic	short	Many roots, fleshy, flexuous.
Ph. mannii	Sikkim, Assam[a]	Unknown	Epiphytic	Very short	Flexuous, glabrous.
Ph. modesta	Malaysia, Borneo	Unknown	Epiphytic	short	Fleshy, flexuous, glabrous.
Ph. cornucervi	Myanmar,Thailand[a]	Unknown	Epiphytic	short	Many roots fleshy, flexuous.
Ph. pantherina	Malaysia, Borneo	Unknown	Epiphytic or litophytic plant.	short	Glabrous.
Ph. lamelligera	Malaysia, Borneo	Unknown	Epiphytic	short	Many roots fleshy, flexuous, glabrous.
Ph. amabilis	Malaysia, Borneo	Unknown	Epiphytic	short	Long, fleshy, glabrous, flexible.
Ph. parishii	Myanmar, Vietnam[a]	Unknown	Epiphytic	Very short	Many roots, fleshy, long, not very flexuous, glabrous.
Ph. lobbi nepal	Myanmar, Nepal[a]	Unknown	Epiphytic	short	Numerous, fleshy, glabrous.
Ph. speciosa	Katchal, Thailand[a]	Unknown	Epiphytic	short	Fleshy, flexuous, smooth.
Ph. lobbi yellow	India ,Bhutan[a]	Unknown	Epiphytic and Lithophytic	short	Numerous, fleshy, glabrous.
Ph. venosa	Indonesia[a]	Unknown	Epiphytic	short	Lengthened, fleshy, flexuous, glabrous.
Ph. violacea blue	Malaysia	Unknown	Epiphytic	short	Flexible roots, glabrous.
Ph. belina	Malaysia, Borneo	Unknown	Epiphytic	short	Flexible roots.
Ph. violacea malaysia	Malaysia	Unknown	Epiphytic	short	Flexible roots.
Ph. violacea witte	Malaysia	Unknown	Epiphytic	short	Flexible roots, glabrous.
Ph. gigantea	Malaysia, Borneo	Unknown	Epiphytic	Very short	Abundant, rather fleshy, glabrous.

[a] These species were collected from Singapore, and the others from Malaysia.

for the RAPD assay in this study. Twelve different decamer primers (Operon Technologies, Inc., USA) were initially screened. Ten of these were chosen for the final analysis (Table 2).

The Polymerase chain reaction (PCR) was carried out in a volume of 25 µl containing 2.5 µl 10x buffer [10 mM Tris HCl], 2 mM $MgCl_2$, 500 µM deoxynucleotide triphosphates (dNTPs), 0.5 units of *Tag* DNA polymerase, and 20 ng of total genomic DNA.

Amplification was performed in a programmable Thermal Controller (MJ Research Inc., USA) for an initial denaturation of 5 min at 94°C, followed by 38 cycles of

Figure 1. *Phalaenopsis* species with various flower colours and shapes. A (*Ph. minho princess*); B (*Ph. violacea witte*); C (*Ph. Venosa*); D (*Ph. Mannii*); E (*Ph. Belina*); F (*Ph. lamelligera*); G (*Ph. modesta*); H (*Ph. cornucervi*); I (*Ph. speciosa*); J (*Ph. leopard prince*); K (*Ph. hieroglyphica*); L (*Ph. maculata*); M (*Ph. violacea blue*); N (*Ph. Amabilis*); O (*Ph. Gigantean*); P (*Ph. pantherina*); Q (*Ph. lobbinepal*); R (*Ph. Parishii*); S (*Ph. lobbi yellow*); T (*Ph. violacea Malaysia*).

Table 2. Details of the 10-mer random primers (Operon Technologies, Inc., USA) screened for this study and degree of polymorphism and information content for 10 RAPD random primers along with their sequences, applied to 20 species of *Phalaenopsis*

Primer number	Primer name	Sequence[a]	No. of polymorphic bands	% G+C	Melting temperature (Tm) (^{0}C)	PIC
1	OPU3	5-CTATGCCGAC-3	54	60	32	0.98
2	OPU8	5-GGCGAAGGTT-3	42	60	32	0.99
3	OPU10	5-ACCTCGGCAC-3	47	70	34	0.98
4	OPU12	5-TCACCAGCCA-3	36	60	32	0.98
5	OPU13	5-GGCTGGTTCC-3	26	70	34	0.99
6	OPU16	5-CTGCGCTGGA-3	33	70	34	0.98
7	OPAW11	5-CTGCCACGAG-3	49	70	43.6	0.99
8	OPAW17	5-TGCTGCTGCC-3	39	70	43.6	0.98
9	OPAW18	5-GGCGCAACTG-3	36	70	43.6	0.98
10	OPG 14	5-GGATGAGACC-3	52	60	39.5	0.99

[a] the sequence primers OPU3, OPU8, OPU10, OPU12, OPU13 and OPU16 previously reported by Goh et al. 2005.

denaturation at 94°C for 30 s, annealing at 38°C for 1 min, and extension for 2 min at 72°C. A final extension for 5 min, at 72°C was included after the last cycle. The amplification products were stored at 4°C before analysis. A negative control reaction, in which DNA template was omitted, was included in every PCR run in order to ensure that no self ampli-fication or DNA contamination occurred. The amplified products were separated in 1.5% agarose gel electrophoresis using 0.5x TAE buffer, and stained with ethidium bromide and visualized on a UV trans-illuminator. The molecular sizes of the amplification products were estimated using 100 bp DNA ladder plus (MBI Fermentas, Lithuania). The number of mono-morphic bands, number of polymorphic bands, and intensity of bands were recorded. Bands on the photos were then scored using Gel-Pro® Analyzer version 3.0 for Windows ™ (Media Cybernetics, Silver Spring MD, and USA). The RAPD bands were represented as '1' (present) and '0' (absent). The PCR was repeated at least twice in order to check reproducibility.

Data analysis

In the genetic relationship study, only distinct, reproducible, well-resolved RAPD fragments in the size range of 599 - 2914 bp (based on mean) were scored as present (1) or absent (0), and from band scores a binary data matrix was constructed. Genetic similarities were calculated among all possible pairs of accessions using all scorable fragments of RAPD markers. A dendogram of genetic relationship was produced by clustering the data with un-weighted pair-group method using arithmetic average (UPGMA). The co-phenetic correlation coefficient was calculated, and the Mantel test (Mantel, 1967) was performed to check the goodness of fit of cluster analysis to the similarity matrix on which it was used. Simple Matching similarity (SM) was also used for closer com-parison with previously published results. All were performed using the NTSYS-pc 2.02 software package (Rohlf, 1998). Boot-strap analysis (1000 replicates) was performed to assess the relative support for different groups and the stability of the dendrogram, using the TREECON software package version 1.3 (Van de per and De wachter, 1994).

The information content of each RAPD marker was computed as $PIC_i=1-\Sigma p_i^2$, where p_i is the frequency of the i^{th} band. The average polymorphic information content (PIC) was calculated for RAPD markers across assay units by applying the formula above as given by Powell et al. (1996). Each DNA fragment visualized within the gel was considered as a single dominant RAPD marker locus. Only polymorphic bands with strong intensity were scored; each marker was identified by the primer combination and the band number as a suffix. Markers with molecular weight lower than 100 bp were excluded from the data matrix. The discrimination power of each RAPD marker was evaluated by the polymorphism information con-tent (PIC). Finally, the partitioning of molecular variance within and among groups and accessions was calculated by the AMOVA technique (Excoffier et al., 1992) in ARLEQUIN software (Schneider et al., 2001). All significance tests were calculated by performing 1023 permutations.

RESULTS

Variation for RAPD markers

The number of polymorphic bands per RAPD assay unit ranged between 26 and 54, with an overall average of 41.4. For each of 10 assay units, PIC value ranged bet-ween 0.98 and 0.99 (Table 2). In addition, the number of

bands varied from 26 (OPU13) to 54 (OPU3) with average of 20.7 bands per assay unit. For separate assay units, PIC values ranged from 0.98 to 0.99 (Table 2). The mean PIC score for all loci was 0.98. The PIC value provides an estimate of discriminatory power of a marker by taking into account not only the number of alleles at locus but also the relative frequencies of these alleles. The distribution of PIC scores were nearly uniform (random) for the 414 polymorphic RAPD markers.

The pre-screening analysis of the 20 selected species of *Phalaenopsis* and 20 RAPD assay units showed that 10 primers generated strong and reproducible amplification products, all of which displayed polymorphism among the species. As highly polymorphic primers were used for analysis, a relatively large number of polymorphic RAPD markers were detected by these primers. Examples of amplification patterns for molecular characterization obtained by RAPD in different genotypes and related species of *Phalaenopsis* are shown in Figure 2.

Genetic Similarities (GS) estimates between replicated samples of the same cultivars of *Phalaenopsis* from 0.028 to 0.960 with an average of 0.680. In addition, genetic similarity values between cultivated genotypes and related species ranged from 0.00 to 0.28, and the mean, minimum and maximum of similarities between 20 *Phalaenopsis* species were 0.04, 0.00 and 0.28, respectively (Table 3).

On the other hand, principle - coordinate and cluster analysis separated accession and *Phalaenopsis* species into three major groups. The first three principle coordinates accounted for 41.56% of the genetic similarity variance. Finally, the phenogram (Figure 3) and principle-coordinate maps show the groups found with both methods.

A total of 414 bands revealed have been polymorphic. The number of RAPD bands detected by each assay unit (primer) depends on primer, sequence and extent of variation in specific genotypes, therefore, the number of bands varied in different accessions.

RAPD polymorphism and power of discrimination

A total of 414 polymorphic fragments were generated with the 10 primers used. The number of markers per assay unit ranged from 18 to 65 with an average of 42.78. The most polymorphic primers were OPU3, OPG 14, OPW11, and OPU10, which produced 54, 52, 49 and 47 markers respectively (Table 2). The distribution of the fragment sizes was skewed towards larger fragments.

This was mainly a result of the fact that fragments smaller than 100 bp were not included.

The distribution power of each marker was estimated by the PIC. Values of PIC ranged between 0.56 and 0.82 (the expected maximum value for a bi-allelic locus), with an average of 0.68. Hence, a large proportion of markers has a high discrimination power. In addition, the discrimination power of each assay unit was estimated by average of the discrimination power of each marker used. $PIC_{av}=\Sigma PIC_i /N$ was calculated, where PIC_i is the PIC value of the i^{th} RAPD marker and N is the number of RAPD markers generated by an assay unit. Loci that are non-polymorphic (PIC = 0) in the germplasm of interest were excluded from this calculation. Because most loci in the study are polymorphic, this average value for a set of markers should only slightly overestimate the true PIC_{av}.

Finally, to provide an objective comparison, matrices of coephenetic values, generated from RAPD data, were compared using the Mantel test; non significant and quite low correlation between the dendrograms was obtained ($r = 0.33$, $p = 0.9741$) after carrying out 250 random permutation with Maxcomp procedure from NTSYS program.

Partition of genetic variation and diversity within and between species

Analysis of molecular variance was separately performed twice, using geographic origin as the grouping criteria. In these cases, AMOVA demonstrated highly significant variation ($p < 0.001$). Genetic variance was found within species as well as among species (Table 4): the variance within species accounted for 98% of the total variance when groups were based on geographic origin, while the population variance contributed only 2.29 for origin. Genetic diversity within species of *Phalaenopsis*, expressed as AMOVA mean square deviations, was positively correlated ($r = 0.95$; $p < 0.001$) with the percentage of polymorphic markers detected per species. The Bartlett's test for population heterosedasticity was highly significant ($B.p = 3.73$, $p < 0.001$) indicating different levels of variability within different species (Table 4).

Genetic diversity, expressed as a co-ancestry coefficient, among the 20 orchid accessions and related species of *Phalaenopsis* ranged from 14 to 186. Co-ancestry coefficients between Malaysia and non-Malaysia species ranged were generally higher than coefficients between other pairs of species of *Phalaenopsis*, showing that Malaysia species of *Phalaenopsis* were highly heterogeneous. Clustering (UPGMA) based on co-ancestry

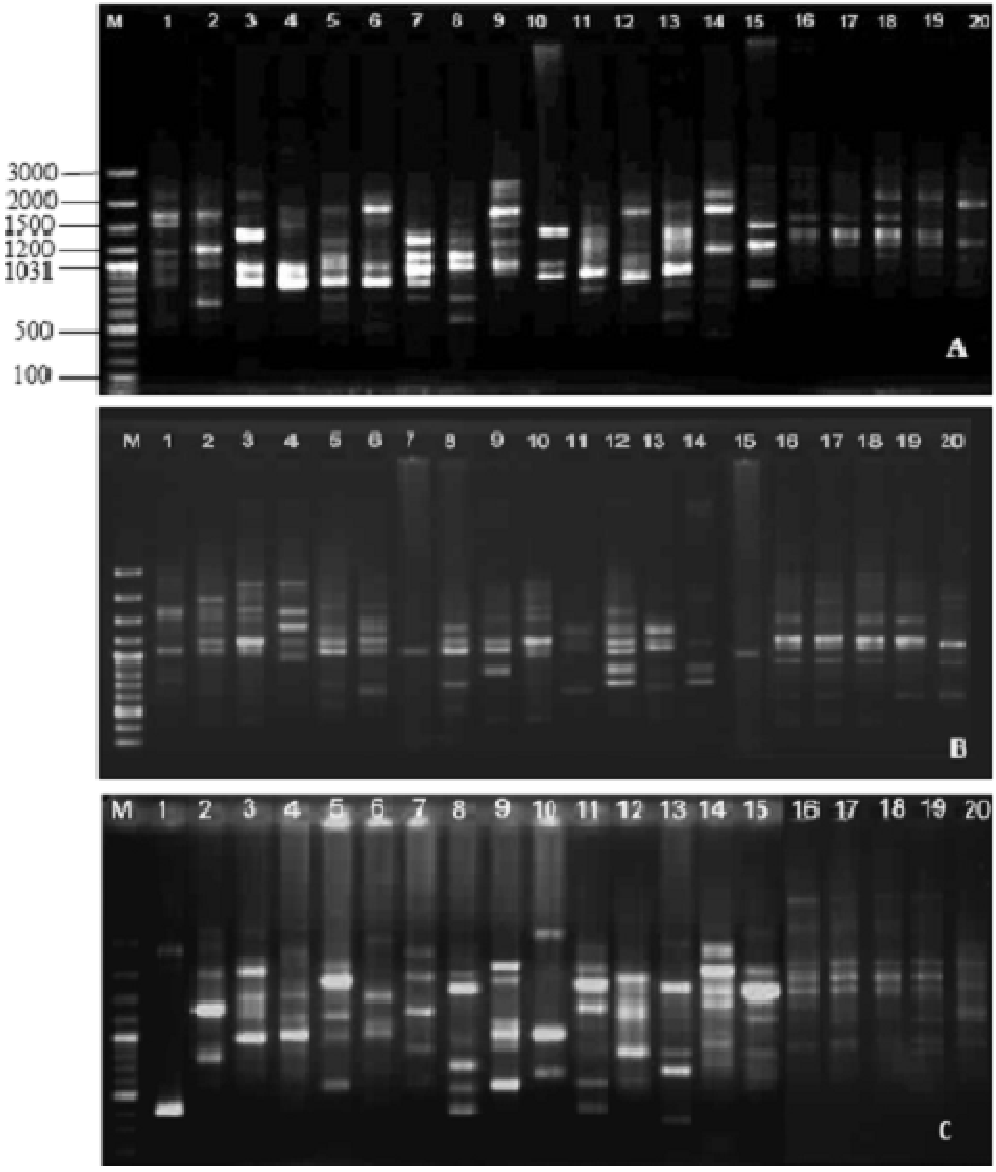

Figure 2. RAPD profiles in agarose gel from 20 species of Phalaenopsis using primers OPU10 (A), OPAW18 (B), and OPU16 (C), respectively. M: molecular size ladder x 100 bp. numbers correspond to the genotypic number listed in Table 1.

co-efficients clearly separated the 20 orchid accessions and related species of *Phalaenopsis* into three clusters: cluster one containing *Ph. violacea blue*, *Ph. belina*, *Ph. violacea malaysia, Ph. violacea witte* and *Ph. gigantea* and cluster two contained *Ph. hieroglyphica, Ph. maculata, Ph. lamelligera, Ph. amabilis, Ph. parishii, Ph. labbi nepal, Ph. speciosa, Ph. lobbi yellow* and *Ph. venosa*. The third cluster consisted of *Ph. minho princess, Ph. leopard prince, Ph. mannii, Ph. modesta, Ph. cornucervi* and *Ph. pantherina*. Clustering of all cultivars based on Jaccard's similarity also clearly grouped all species of *Phalaenopsis* in three clusters (Figure 4).

Table 3. Jacard similarity coefficients matrix for 20 *Phalaenopsis* based on RAPD data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.00																			
2	0.25	1.00																		
3	0.03	0.03	1.00																	
4	0.03	0.03	0.08	1.00																
5	0.06	0.02	0.08	0.04	1.00															
6	0.12	0.13	0.03	0.04	0.10	1.00														
7	0.05	0.02	0.05	0.02	0.13	0.12	1.00													
8	0.09	0.04	0.06	0.02	0.13	0.06	0.10	1.00												
9	0.03	0.02	0.07	0.05	0.07	0.04	0.01	0.08	1.00											
10	0.01	0.06	0.11	0.06	0.06	0.03	0.00	0.05	0.02	1.00										
11	0.01	0.03	0.03	0.02	0.05	0.08	0.04	0.07	0.05	0.07	1.00									
12	0.00	0.01	0.07	0.04	0.06	0.01	0.01	0.05	0.09	0.05	0.08	1.00								
13	0.02	0.02	0.03	0.04	0.06	0.03	0.04	0.02	0.03	0.05	0.12	0.11	1.00							
14	0.04	0.02	0.06	0.06	0.05	0.06	0.05	0.02	0.04	0.02	0.05	0.09	0.02	1.00						
15	0.04	0.03	0.08	0.04	0.05	0.01	0.02	0.02	0.04	0.08	0.09	0.06	0.07	0.06	1.00					
16	0.04	0.04	0.02	0.04	0.03	0.04	0.01	0.02	0.03	0.00	0.01	0.05	0.02	0.02	0.01	1.00				
17	0.02	0.00	0.05	0.01	0.04	0.01	0.04	0.05	0.02	0.00	0.01	0.01	0.01	0.02	0.01	0.22	1.00			
18	0.01	0.02	0.01	0.00	0.03	0.01	0.02	0.03	0.00	0.00	0.03	0.01	0.01	0.00	0.01	0.09	0.22	1.00		
19	0.04	0.05	0.01	0.01	0.07	0.02	0.04	0.02	0.01	0.01	0.02	0.01	0.05	0.02	0.02	0.13	0.14	0.28	1.00	
20	0.05	0.03	0.03	0.00	0.02	0.05	0.01	0.04	0.02	0.02	0.04	0.02	0.05	0.02	0.06	0.11	0.04	0.02	0.04	1.00

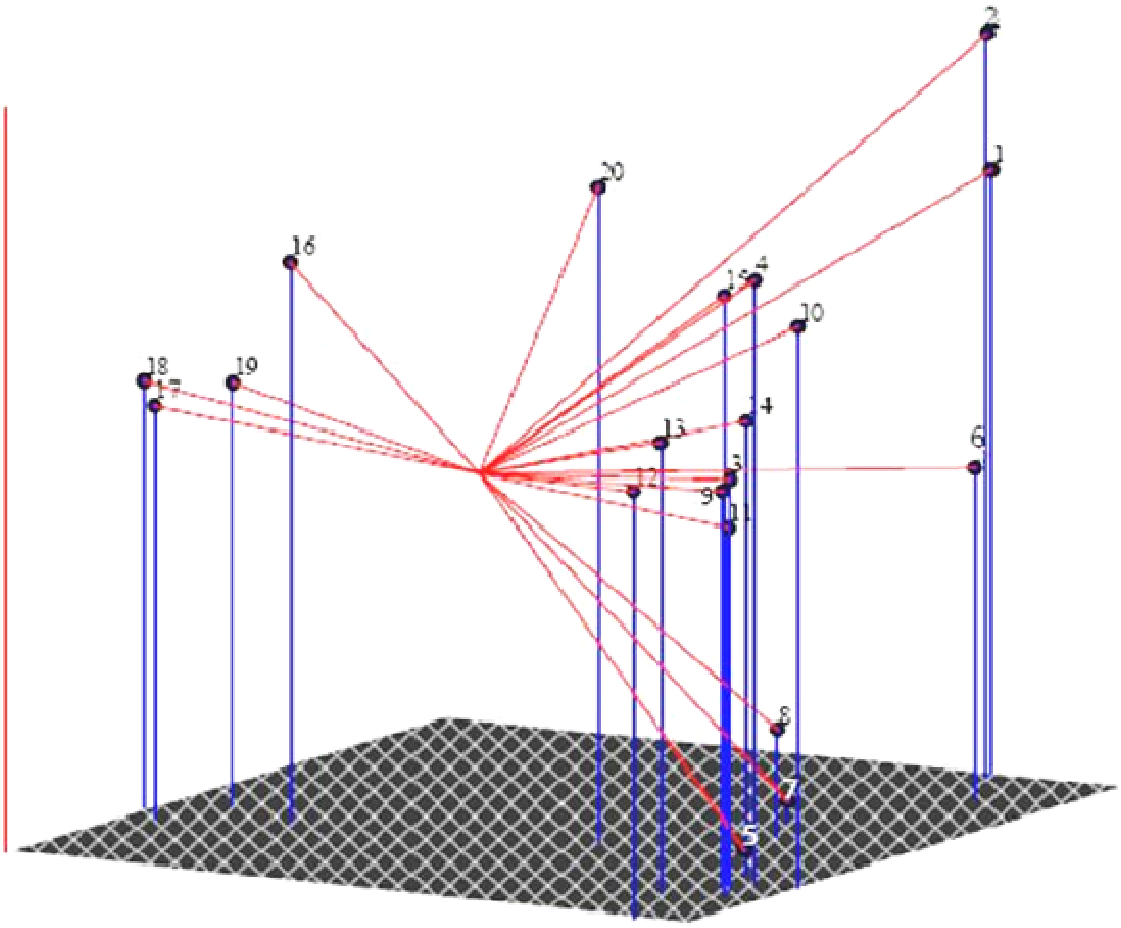

Figure 3. Genetic relationships depicted among 20 orchid accessions and related species of *Phalaenopsis* by the first three components (PC1, PC2, and PC3) derived from principal coordinate analysis of RAPD data.

Genetic similarity and clustering

The similarity coefficients for the 20 orchid accessions and related species of *Phalaenopsis* varied from a maximum of 0.28 (between *Ph. violacea malaysia* and *Ph. violacea witte*) to a minimum of 0.00 (between *Ph. minho princess, Ph. labbi nepal, Ph. belina, Ph. leopard prince, Ph. violacea malaysia, Ph. maculata, Ph. gigantea, Ph. amabilis, Ph. cornucervi, Ph. lamelligera, Ph. venosa, Ph. violacea blue, Ph. amabilis* and *Ph. lobbi yellow*), with average of 0.04, indicating the high level of genetic variation that exists in the species of *Phalaenopsis*. The Mantel method used for comparing the similarity matrixes produced correlation coefficients that were statistically significant for RAPD markers. The co-phenetic correlation coefficient between dendrogram and the original similarity matrix for RAPD was large and significant ($r = 0.87$, $t = 11.75$), giving a good degree of confidence in the association obtained for the accessions.

The dendrograms (Figure 4) reflect relationships among many of the 20 orchid accessions and related species of *Phalaenopsis* in ways that depend on their area of diffusion and/or pedigree information. The RAPDs discriminated most species of *Phalaenopsis* effectively and separated. Another important aspect in cluster analysis is to determine the optimal number of clusters or number of acceptable clusters. In essence, this involves deciding where to "cut" a dendrogram to find the true or natural groups. An "acceptable cluster" is defined as "a group of two or more genotypes where the within-cluster

Table 4. Analysis of molecular variance (AMOVA) for 20 orchid accessions and related species of *Phalaenopsis* based on 414 RAPD markers.

Source of variance	df	Sum of square	Variance component	%of variation	Significance[a]
		Groups based on geographical origin b			
Variance among population	1	44.133	0.86464	2.29	P<0.001
Variance within population	18	663.667	36.87.37	97.71	P<0.001
total	19	770.800	37.73501		

[a] Significance of variance component expressed as the probability of obtaining a more extreme random value computed from nonparametric procedures (1000 data permutation).
[b] Two groups consist of species from Malaysia and Singapore based on geographical distribution.

genetic distance is lower than the overall mean genetic distance and the between cluster distances are greater than the within-cluster distance of both cluster involved (Brown-Guedira et al., 2000; Sorkheh et al., 2007). Some relatively simple ways of finding the optimal number of clusters are the D^2, upper tail approach (Wishart, 1987) and statistical techniques such as bootstrapping, MANOVA (Multivariate Analysis of Variance), and or discriminate analysis. We used the MANOVA method (Sorkheh et al., 2007), where the optimal number of clusters or groups occurs when the F-value is highest. In our study, this cutting point is 0.45.

The dendrogram consists of three well-supported clusters, that is, three groups of plants: *Ph. violacea blue, Ph. belina, Ph. violacea malaysia, Ph. violacea witte* and *Ph. gigantea* (cluster I); and *Ph. hieroglyphica, Ph. maculata, Ph. lamelligera, Ph. amabilis, Ph. parishii, Ph. labbi nepal, Ph. speciosa, Ph. lobbi yellow and Ph. venosa* (cluster II). Cluster III contains *Ph. minho princess, Ph. leopard prince, Ph. mannii, Ph. modesta, Ph. cornucervi* and *Ph. pantherina*. The strength of relationships varied, as assessed by bootstrapping analysis. In the dendrogram, there is very strong support for clustering of species that were closely related by pedigree or origin. As shown in Figure 4, the species of *Ph. Gigantean,* which belongs to section Ambo-inenses, separates from other species with a coefficient of 0.93. The species of *Ph. violacea blue, Ph. belina, Ph. violacea malaysia* and *Ph. violacea witte*, which belongs to Zebrine section, separates from other species with a coefficient of 0.85. The second group separated from others into subgroups with a coefficient of 0.94.

The third group consists of *Ph. minho princess, Ph. leopard prince, Ph. mannii, Ph. modesta, Ph. cornucervi* and *Ph. pantherina*. It separated from other species with a coefficient of 0.93. The lower similarity indices and more divergent dendrogarm branch points of 20 orchid accessions and related species of *Phalaenopsis* demonstrate the high genetic variability of the study material. *Ph. minho princess and Ph. leopard princer* were obtained from a cross *Phal Sun Prince* × *Phal Ta Lin Freeds* and *Dtps Sun Prince* × *Phal Ho's French Fantasia* respectively, and they grouped into the same subcluster in the resultant dendrogram.

DISCUSSION

RAPDs proved to be a powerful tool for the molecular characterization of the 20 orchid accessions and related species of *Phalaenopsis*, and they are also useful for producing genetic maps and marker-assisted selection in crop plants. In general, our RAPD data are in agreement with the classifications based on morphological characters proposed by Sweet (1980) and Christenson (2001), respectively. In accord with the report of Goh et al. (2005), the limited number of RAPD primers used in our analysis generated sufficient variability to differentiate the different subgenera and even species of *Phalaenopsis*.

RAPD analyses are performed using low stringency conditions. Hence, mismatches can occur between the primer and its target sequence in amplification reactions (MacPherson et al., 1993; Chi-chu and Chang-Hung,

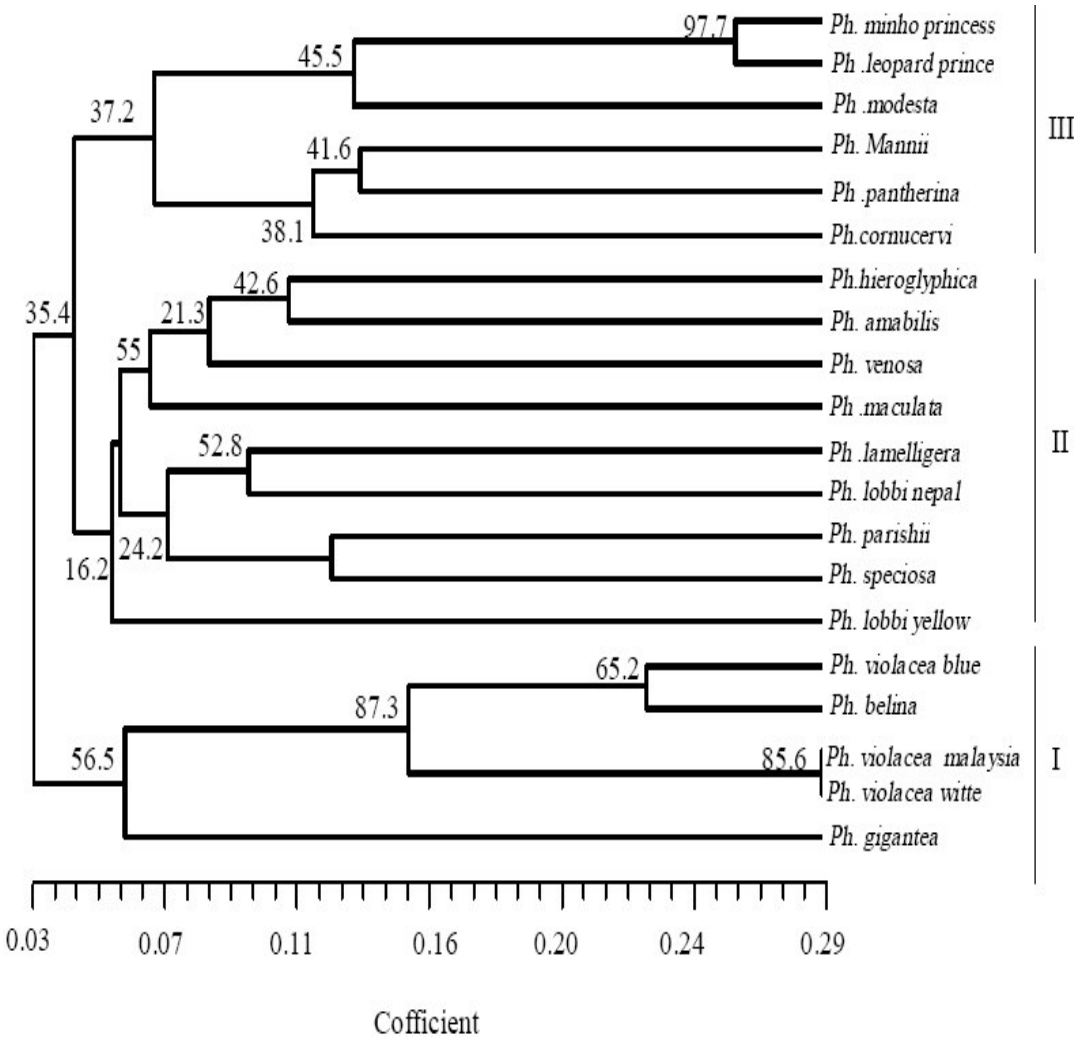

Figure 4. Dendrogram obtained with the similarity Jacard coefficient pair group method using arithmetic average (UPGMA) clustering algorithm from 414 RAPD markers for 20 orchid accessions and related species of *Phalaenopsis*. The value on the dendrogram gives the stability of nodes estimated with a bootstrap procedure

2007). Different thermal cyclers (Edwards, 1998), temperature profiles, band of DNA polymerases (Schierwater and Ender, 1993) and the concentration of $MgCl_2$, primer and template DNA can affect the reproducibility of the RAPD assay (Muralidharan and Wakeland, 1993). In our work, we standardised all of the above parameters prior to performing our analyses. Another limitation of RAPD markers is that they can only detect dominant inheritance (Devos and Gale, 1992; Choi et al., 2006).

The significantly greater than the mean PIC we estimated for RAPDs was (0.98). The minimum PIC scores for a RAPD marker (or any bi-allelic marker) is 0.5, whereas the maximum PIC scores for and RFLP marker is 1.0. Thus for example, when a RAPD fragment is present in half and missing in half of accessions and related species of *Phalaenopsis*, the PIC score is 0.5. Roughly 95% of the RAPD fragments in our study had maximum PIC scores. The PIC scores for an RFLP marker can be increased by testing additional restriction enzymes.

The results of this study are consistent with an earlier

study of species of *Phalaenopsis* for RAPD marker (Fu et al., 1997; Sun and Wong, 2001; Been et al., 2002; Feng et al., 2003; Goh et al., 2005; Ching-Chun et al., 2006; Wen-Huei et al., 2006), where accessions and related species of *Phalaenopsis* were also strongly separated into groups. These groups reflected the fundamental heterotic patterns of *Phalaenopsis* and the widespread practice of producing new accessions by crossing species of *Phalaenopsis* for improvement of orchid. We found, like Goh et al. (2005) and Been et al. (2002), a clear grouping of different species of *Phalaenopsis* according to classification in different section.

The range of the amplified bands' sizes in accessions and related species of *Phalaenopsis* was also similar to those reported by Goh et al. (2005) for moth orchids and by Been et al. (2002) for species of *Phalaenopsis* in studies using the same primer pairs. Variation in the numbers of polymorphic RAPD markers and the total number of polymorphic bands were observed (Table 3), allowing differentiation into two groups of species: one group having high numbers of polymorphic bands (*P. minho princess, Ph. leopard prince, Ph. hieroglyphica, Ph. maculata, Ph. mannii, Ph. modesta, Ph. pantherina, Ph. lamelligera, Ph. parishii, Ph. labbi nepal, Ph. speciosa, Ph. lobbi yellow* and *Ph. venosa*), the other group with low numbers of polymorphic bands (*P. cornucervi, Ph. amabilis, Ph. violacea blue, Ph. belina, Ph. violacea malaysia, Ph. violacea witte* and *Ph. gigantea*). Differences in amplification suc-cess for RAPD markers were observed among species of *Phalaenopsis*. Result also demonstrated the possibility of cross-species and consequently the value of markers developed in species of *Phalaenopsis* for molecular characterization of other species within the subgenus. Successful hybridization between Phal Sun Prince × Phal Ta Lin Freeds (*P. minho princess*), and Dtps Sun Prince × Phal Ho's French Fantasia (*P. leopard prince*) has also been reported by different researchers (Sweet, 1980; Christenson, 2001; Chen and Chen, 2003; Shipunov et al., 2005; Taywiya et al., 2008).

Both accessions and related species of *Phalaenopsis* suffer from limited gene pool availability for future breeding progress. Inter-specific gene transfer among these *Phalaenopsis* species offers a greatly expanded genetic diversity for breeders, particularly given the relative ease of initial hybridization and subsequent backcrossing (Fu et al., 1997; Fu et al., 2002; Chang, 2008). Hence, further RAPD analysis of this germplasm offers opportunities for determining more precisely genetic relationships and molecular characterization and could be an important tool for marker assisted gene transfer. DNA fingerprinting and molecular characterization using RAPD analysis could also be very useful for the selection of the most promising progeny from inter-specific crosses or back crosses, leading to greatly improved breeding efficiency. Goh et al. (2005) reported that in all accessions, intra-specific similarity was higher than inter-specific similarity and among all the accessions, the range of similarity coefficients was from 0.232 to 0.992. In addition, Goh et al. (2005) also reported that between accessions, *Ph. pulchra* accession no. 452 and *Ph. reichenbachiana* accession no. 342 have the highest similarity coefficient of 0.848. *Ph. chibae* accession no. 568 and *Ph. cochlearis* accession no. 277 exhibited the lowest similarity coefficient of 0.232.

The species of *Ph. minho princess* and *Ph. leopard prin-cess* grouped within the same cluster. The presence of these species in the same cluster is easily explained due to two reasons: First, *Ph. minho princess* was obtained from a cross between *Ph. Sun Prince* × *Ph. Ta Lin Freeds*, and *Ph. leopard princess* was obtained from a cross between *Dtps Sun Prince* × *Phal Ho's French Fantasia*. Secondly, this species may have grouped into section Doritaenopsis due to its floral morphological resemblance to its putative parent plants. Furthermore, a photograph recently published in the Orchid Digest volume 66(4), of a hybrid between *Ph. minho princess* and *Ph. leopard princess* resembles morphologically. Using RAPD analysis, we were able to conclude that *Ph. minho princess* and *Ph. leopard princess* were a hybrid. The clustering strategy also did place *Ph. minho princess* and *Ph. leopard princess* in the same cluster (Cluster III) near *Ph. modesta* from section Amboinenses (Figure 4). The species of *Ph. mannii, Ph. cornucervi,* and *Ph. pantherina* are found in Cluster III together with species found in sections Doritaenopsis and Amboinenses. Both species of *Ph. mannii* and *Ph. pantherina* belong to section Polychilos and based on floral morphology, have two pollinia and star-shaped flowers. *Ph. cornucervi* is also clustered next to each other in the dendrogram and found in section Polychilos and based on floral morphology, have star-shaped, yellow, large and round flowers that are found in Cluster III.

P. cornucervi is widespread in distribution and is, in the view of Christenson (2001), phenotypically highly variable. In our study, *Ph. cornucervi* from Myanmar, Thailand, are not clustered next to those from Borneo. More sampling and/or population studies of the plants from the various localities should be undertaken to resolve this apparent inconsistency with previous reports

(Goh et al., 2005; Chen et al., 2005).

RAPD markers showed that species of *Ph. violacea blue, Ph. belina, Ph. violacea malaysia, Ph. violacea witte* and *Ph. Gigantea* that were clustered together, are found in Cluster I, sections Zebrinae and Amboionenses, respectively. The species of *Ph. gigantea* separated from other species of *Phalaenopsis*, based on floral morphology, have been star - shaped with spotted flowers; but other species in cluster I have star-shaped, fragmented flowers and also belonging to section Zebrinae (Figures 1 and 4).

Out of the three clusters obtained, only two of our clusters correspond to work done by Fu et al. (1997) and/or Been et al. (2002). In Fu et al. (1997), *Ph. mannii* was clustered together with taxa found in section Amboinenses, which contradicts our data in which *Ph. mannii* was clustered together with the rest of section/subgenus Polychilos. We suspect that the interpretation of this placement was due to the lack of sampling in their work, since *Ph. mannii* was the only representative taxon from section Polychilos that is corresponding with previous report by Goh et al. (2005).

Been et al. (2002) obtained eight clusters from RAPD markers. In six of these clusters, taxa from various sections/subgenera of Sweet (1980) and Christenson (2001) were found clustered together, for example, in Cluster II of Been et al. (2002), *Ph. amabilis, Ph. bellina* and *Ph. mannii* were clustered together. When classified using morphological data (Sweet, 1980; Christenson, 2001), all of these species belong to different sections/subgenera. Been et al. (2002) postulated that the differences obtained via traditional morphological classification and RAPD data could be due to morphological modifications by regional and environmental changes. It should be noted that plant materials used by Been et al. (2002) were obtained from a commercial orchid company. There fore, the authenticity of the plant materials that they worked on is doubtful since *Phalaenopsis* is a genus that is commonly utilized for breeding purposes. Furthermore, it was pointed out that herbarium specimens should have been examined for a more reliable identification of the species (Goh et al., 2005).

Besse et al. (2004) reported that the level of intraspecific variation detected with RAPD markers in cultivars of the *Vanilla,* one of the orchidaceae, was low. However, polymorphic bands from RAPD analysis of *Phalaenopsis* composed 95% of the total bands in this study. This result indicated that high levels of polymorphisms in the two *Phalaenopsis* spp. examined and that the technique could possibly differentiate *Phalaenopsis* spp. Identification of *Phalaenopsis* has been previously investigated using RAPD. Goh et al. (2005) reported that the genetic distance and relationships of 149 accessions representing 46 species in the genus *Phalaenopsis* and four species in Paraphalaenopsis were studied using random amplified polymorphic DNA (RAPD) markers and that a high genetic diversity existed. They reported that the extensive genetic variation among the present *Phalaenopsis* cultivars was a result of the breeding program.

Our study clearly demonstrated that RAPD markers are useful in unambiguous separation of the genus into three clusters and is, therefore, a useful tool for identifying *Phalaenopsis* orchids up to the specific and/or subgeneric levels. For more reliable classification of the species, herbarium specimens of every known species need to be compared as well as more species and ecotypes of many regions. With genetic distances of species obtained from this study, plant breeders can be better informed of the potential rates of success of their breeding experiments. RAPDs can be usefully applied to distinguish *Phalaenopsis* species, even with a relatively low number of primers.

Conclusion

RAPD results in this study clearly indicated that cultivars or intra-species of *Phalaenopsis* could be differentiated from each other and that classification using RAPD agreed well with ecological, physiological and morphological based classifications. Although RAPD markers have been successfully employed to reveal relationships and classifications of the *Phalaenopsis* (Been et al., 2003; Goh et al., 2005), this study shows that RAPD markers based on the genomic DNA of *Phalaenopsis* provided phylogenetic information that addresses the genetic relationship of inter-/intra-species oriental cymbidiums. The discriminatory band patterns and phylogenetic tree created from the results of this study were successfully used to determine oriental *Phalaenopsis* lineages.

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