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Random amplified polymorphic DNA analysis of the moth orchids, *Phalaenopsis* (Epidendroideae: Orchidaceae)

M.W.K. Goh[1], P.P. Kumar[1], S.H. Lim[2] & H.T.W. Tan[1,*]
[1]*Department of Biological Sciences, Faculty of Science, National University of Singapore, 14 Science Drive 4, Singapore 117543, Republic of Singapore;* [2]*Biotechnology Program, Malaysia University of Science and Technology, GL33, Ground Floor, Block C, Dataran Usahawan Kelana, 17 Jalan SS7/26, 47301 Petaling Jaya, Selangor, Malaysia; (*author for correspondence: e-mail: dbsttw@nus.edu.sg)*

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Summary

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. The genus *Paraphalaenopsis* was used as an outgroup. A total of 20 random primers were screened and out of these, six random primers provided 123 polymorphic bands and zero monomorphic bands. Pairwise genetic distances between accessions were estimated according to Nei and Li (1979). Cluster analysis of data using the UPGMA algorithm placed the species in seven groups that are mostly congruent with those based on morphological characters erected by previous workers. As observed from the banding patterns, *Ph. doweryensis*, which is suspected to be a hybrid of *Ph. gigantea* and *Ph. kunstleri* or *Ph. cochlearis,* is not. RAPD markers can thus be successfully applied in this economically important group of orchids for the study of relationships and to distinguish taxa up to the specific level.

Introduction

The genus *Phalaenopsis* belongs to the family Orchidaceae, subfamily Epidendroideae, tribe Vandeae and subtribe Aeridinae (Dressler, 1993). Its natural distribution is from India, through South-East Asia to the Philippines, New Guinea and Australia (Sweet, 1980). 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). *Phalaenopsis* plants have short stems usually with three to six leaves. The leaf blades are usually longer than broad (Batchelor, 1982), 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).

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 *Phalaenopsis* 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 & 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, other than morphological characters, such as RAPD markers. RAPD analysis is a fingerprinting method using short, random, oligonucleotide primers to search for variation in the entire genomic DNA (Williams et al., 1990) 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, e.g., *Goodyera procera* (Wong & Sun, 1999); *Eulophia sinensis, Zeuxine gracilis*, and *Zeuxine strateumatica* (Sun & Wong, 2001) and *Changnienia amoena, Paphiopedilum malipoense* and *Paphiopedilum micranthum* (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 *Vanda* and Benner et al. (1995) detected high levels of inter- and infraspecific 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).

In the current study, we hope to (1) differentiate between the different species of *Phalaenopsis* using RAPD molecular markers, (2) determine the relationships of the species within the genus using cluster analysis and (3) determine the relationships of the genus with *Doritis*, *Kingidium* and the outgroup *Paraphalaenopsis*.

Materials and methods

To determine the intergeneric relationships of the genus *Phalaenopsis*, 149 accessions from 46 species of *Phalaenopsis* and four species of *Paraphalaenopsis* were analyzed. Most of the species in the genus *sensu* Christenson (2001) were included. We sampled one to several representatives of each section/subgenus of *Phalaenopsis* in this study. 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 infraspecific variation be detected, if present. Independent extraction of DNA from each accession was carried out to determine repeatability of the results in repeated analyses using the same accessions.

Plant materials

Tissue samples used in this study were obtained from wild plants that were collected from wild forests and/or similar plants that were subsequently cultivated. A list of the species sampled and their accession numbers is provided (Table 1). The genus *Paraphalaenopsis* was used as the outgroup. Healthy leaves or flowers were collected from each plant. The plant samples were first washed with tap water and then 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 aluminium foil and stored at −80 °C or used immediately for DNA extraction.

All voucher specimens were deposited at the Herbarium, Raffles Museum of Biodiversity Research, Department of Biological Sciences, National University of Singapore (SINU), for future reference. All floral specimens were preserved in 4% formaldehyde (w/v). Major herbaria were visited and specimens were borrowed (see acknowledgements for list of herbaria) to examine all the type specimens so as to verify the identity of the accessions used in this study. A morphological study using cladistic analyses will be published elsewhere.

Table 1. List of orchid species sampled, their corresponding accession numbers, classification according to Christenson (2001) and the cluster to which they belong to in the dendrogram (Figure 2)

Taxon name	Accession no.	Classification (Section/subgenus)	Cluster no.
Genus *Phalaenopsis*			
Ph. lowii Rchb.f.	M.W.K. Goh P326	*Proboscidioides*	1
Ph. lowii Rchb.f.	M.W.K. Goh P550	*Proboscidioides*	1
Ph. honghenensis F.Y. Liu	M.W.K. Goh P594	*Aphyllae*	1
Ph. taenialis (Lindl.) Christenson & Pradhan[a]	M.W.K. Goh P261	*Aphyllae*	1
Ph. taenialis (Lindl.) Christenson & Pradhan[a]	M.W.K. Goh P262	*Aphyllae*	1
Ph. appendiculata Carr[a]	M.W.K. Goh P592	*Parishianae*	1
Ph. minus (Seidenf.) Christenson[a]	M.W.K. Goh P259	*Aphyllae*	1
Ph. minus (Seidenf.) Christenson[a]	M.W.K. Goh P341	*Aphyllae*	1
Ph. minus (Seidenf.) Christenson[a]	M.W.K. Goh P409	*Aphyllae*	1
Ph. gibbosa H.R. Sweet	M.W.K. Goh P116	*Parishianae*	1
Ph. gibbosa H.R. Sweet	M.W.K. Goh P403	*Parishianae*	1
Ph. gibbosa H.R. Sweet	M.W.K. Goh P404	*Parishianae*	1
Ph. parishii Rchb.f.[a]	M.W.K. Goh P265	*Parishianae*	1
Ph. pantherina Rchb.f.	M.W.K. Goh P499	*Polychilos*	2
Ph. pantherina Rchb.f.	M.W.K. Goh P500	*Polychilos*	2
Ph. pantherina Rchb.f.	M.W.K. Goh P237	*Polychilos*	2
Ph. pantherina Rchb.f.	M.W.K. Goh P41	*Polychilos*	2
Ph. pantherina Rchb.f.	M.W.K. Goh P109	*Polychilos*	2
Ph. pantherina Rchb.f.	M.W.K. Goh P144	*Polychilos*	2
Ph. cornucervi (Breda) Bl. & Rchb.f.	M.W.K. Goh P2	*Polychilos*	2
Ph. cornucervi (Breda) Bl. & Rchb.f.	M.W.K. Goh P3	*Polychilos*	2
Ph. cornucervi (Breda) Bl. & Rchb.f.	M.W.K. Goh P4	*Polychilos*	2
Ph. mannii Rchb.f.	M.W.K. Goh P243	*Polychilos*	2
Ph. mannii Rchb.f.	M.W.K. Goh P471	*Polychilos*	2
Ph. mannii Rchb.f.	M.W.K. Goh P472	*Polychilos*	2
Ph. cornucervi (Breda) Bl. & Rchb.f.	M.W.K. Goh P28	*Polychilos*	2
Ph. cornucervi (Breda) Bl. & Rchb.f.	M.W.K. Goh P192	*Polychilos*	2
Ph. cornucervi (Breda) Bl. & Rchb.f.	M.W.K. Goh P193	*Polychilos*	2
Ph. cochlearis Holttum	M.W.K. Goh P66	*Fuscatae*	2
Ph. cochlearis Holttum	M.W.K. Goh P241	*Fuscatae*	2
Ph. kunstleri Hook.f.	M.W.K. Goh P82	*Fuscatae*	2
Ph. kunstleri Hook.f.	M.W.K. Goh P136	*Fuscatae*	2
Ph. kunstleri Hook.f.	M.W.K. Goh P153	*Fuscatae*	2
Ph. cochlearis Holttum	M.W.K. Goh P277	*Fuscatae*	2
Ph. kunstleri Hook.f.	M.W.K. Goh P391	*Fuscatae*	2
Ph. viridis J.J. Sm	M.W.K. Goh P420	*Fuscatae*	2
Ph. amboinensis J.J. Sm	M.W.K. Goh P6	*Amboinenses*	3
Ph. amboinensis J.J. Sm	M.W.K. Goh P30	*Amboinenses*	3
Ph. amboinensis J.J. Sm	M.W.K. Goh P57	*Amboinenses*	3
Ph. floresensis Fowlie	M.W.K. Goh P121	*Amboinenses*	3
Ph. floresensis Fowlie	M.W.K. Goh P236	*Amboinenses*	3

(*Continued on next page*)

Table 1. (*Continued*)

Taxon name	Accession no.	Classification (Section/subgenus)	Cluster no.
Ph. floresensis Fowlie	M.W.K. Goh P211	*Amboinenses*	3
Ph. bastianii Gruss & Röllke	M.W.K. Goh P431	*Amboinenses*	3
Ph. bellina (Rchb.f.) Christenson	M.W.K. Goh P1	*Amboinenses*	3
Ph. bellina (Rchb.f.) Christenson	M.W.K. Goh P9	*Amboinenses*	3
Ph. bellina (Rchb.f.) Christenson	M.W.K. Goh P10	*Amboinenses*	3
Ph. fimbriata J.J. Sm	M.W.K. Goh P535	*Amboinenses*	3
Ph. fimbriata J.J. Sm.	M.W.K. Goh P536	*Amboinenses*	3
Ph. hieroglyphica (Rchb.f.) H.R. Sweet	M.W.K. Goh P425	*Amboinenses*	3
Ph. hieroglyphica (Rchb.f.) H.R. Sweet	M.W.K. Goh P438	*Amboinenses*	3
Ph. hieroglyphica (Rchb.f.) H.R. Sweet	M.W.K. Goh P519	*Amboinenses*	3
Ph. javanica J.J. Sm.	M.W.K. Goh P204	*Amboinenses*	3
Ph. javanica J.J. Sm.	M.W.K. Goh P446	*Amboinenses*	3
Ph. javanica J.J. Sm.	M.W.K. Goh P533	*Amboinenses*	3
Ph. lueddemanniana Rchb.f.	M.W.K. Goh P5	*Amboinenses*	3
Ph. lueddemanniana Rchb.f.	M.W.K. Goh P123	*Amboinenses*	3
Ph. lueddemanniana Rchb.f.	M.W.K. Goh P125	*Amboinenses*	3
Ph. mariae Burb. ex R. Warner & B.S. Williams	M.W.K. Goh P424	*Amboinenses*	3
Ph. mariae Burb. ex R. Warner & B.S. Williams	M.W.K. Goh P565b	*Amboinenses*	3
Ph. mariae Burb. ex R. Warner & B.S. Williams	M.W.K. Goh P565a	*Amboinenses*	3
Ph. micholitzii Rolfe	M.W.K. Goh P139	*Amboinenses*	3
Ph. micholitzii Rolfe	M.W.K. Goh P347	*Amboinenses*	3
Ph. micholitzii Rolfe	M.W.K. Goh P448	*Amboinenses*	3
Ph. pulchra (Rchb.f.) H.R. Sweet	M.W.K. Goh P364	*Amboinenses*	3
Ph. pulchra (Rchb.f.) H.R. Sweet	M.W.K. Goh P433	*Amboinenses*	3
Ph. pulchra (Rchb.f.) H.R. Sweet	M.W.K. Goh P452	*Amboinenses*	3
Ph. reichenbachiana Rchb.f. & Sander	M.W.K. Goh P113	*Amboinenses*	3
Ph. reichenbachiana Rchb.f. & Sander	M.W.K. Goh P342	*Amboinenses*	3
Ph. reichenbachiana Rchb.f. & Sander	M.W.K. Goh P418	*Amboinenses*	3
Ph. modesta J.J. Sm.	M.W.K. Goh P25	*Amboinenses*	3
Ph. modesta J.J. Sm.	M.W.K. Goh P78	*Amboinenses*	3
Ph. modesta J.J. Sm.	M.W.K. Goh P212	*Amboinenses*	3
Ph. pallens (Lindl.) Rchb.f.	M.W.K. Goh P143	*Amboinenses*	3
Ph. pallens (Lindl.) Rchb.f.	M.W.K. Goh P240	*Amboinenses*	3
Ph. pallens (Lindl.) Rchb.f.	M.W.K. Goh P344	*Amboinenses*	3
Ph. venosa Shim & Fowlie	M.W.K. Goh P54	*Amboinenses*	3
Ph. venosa Shim & Fowlie	M.W.K. Goh P257	*Amboinenses*	3
Ph. venosa Shim & Fowlie	M.W.K. Goh P263	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P45	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P141	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P142	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P18	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P47	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P150	*Amboinenses*	3

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Table 1. (*Continued*)

Taxon name	Accession no.	Classification (Section/subgenus)	Cluster no.
Ph. violacea Witte	M.W.K. Goh P14	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P46	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P19	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P32	*Amboinenses*	3
Ph. violacea Witte	M.W.K. Goh P93	*Amboinenses*	3
Ph. corningiana Rchb.f.	M.W.K. Goh P174	*Zebrinae*	3
Ph. corningiana Rchb.f.	M.W.K. Goh P175	*Zebrinae*	3
Ph. corningiana Rchb.f.	M.W.K. Goh P368	*Zebrinae*	3
Ph. sumatrana Korth & Rchb.f.	M.W.K. Goh P62	*Zebrinae*	3
Ph. sumatrana Korth & Rchb.f.	M.W.K. Goh P177	*Zebrinae*	3
Ph. sumatrana Korth & Rchb.f.	M.W.K. Goh P169	*Zebrinae*	3
Ph. inscriptiosinensis Fowlie	M.W.K. Goh P254	*Zebrinae*	3
Ph. inscriptiosinensis Fowlie	M.W.K. Goh P324	*Zebrinae*	3
Ph. inscriptiosinensis Fowlie	M.W.K. Goh P497	*Zebrinae*	3
Ph. tetraspis Rchb.f.	M.W.K. Goh P29	*Zebrinae*	3
Ph. tetraspis Rchb.f.	M.W.K. Goh P50	*Zebrinae*	3
Ph. tetraspis Rchb.f.	M.W.K. Goh P51	*Zebrinae*	3
Ph. doweryensis Garay & Christenson	M.W.K. Goh P351	*Amboinenses*	4
Ph. doweryensis Garay & Christenson	M.W.K. Goh P411	*Amboinenses*	4
Ph. doweryensis Garay & Christenson	M.W.K. Goh P467	*Amboinenses*	4
Ph. maculata Rchb.f.	M.W.K. Goh P513	*Amboinenses*	4
Ph. maculata Rchb.f.	M.W.K. Goh P514	*Amboinenses*	4
Ph. gigantea J.J. Sm.	M.W.K. Goh P88	*Amboinenses*	4
Ph. gigantea J.J. Sm.	M.W.K. Goh P137	*Amboinenses*	4
Ph. gigantea J.J. Sm.	M.W.K. Goh P157	*Amboinenses*	4
Ph. amabilis (L.) Bl.	M.W.K. Goh P8	*Phalaenopsis*	5
Ph. amabilis (L.) Bl.	M.W.K. Goh P85	*Phalaenopsis*	5
Ph. amabilis (L.) Bl.	M.W.K. Goh P101	*Phalaenopsis*	5
Ph. equestris (Schauer) Rchb.f.	M.W.K. Goh P15	*Stauroglottis*	5
Ph. equestris (Schauer) Rchb.f.	M.W.K. Goh P33	*Stauroglottis*	5
Ph. equestris (Schauer) Rchb.f.	M.W.K. Goh P36	*Stauroglottis*	5
Ph. celebensis H.R. Sweet	M.W.K. Goh P112	*Stauroglottis*	5
Ph. celebensis H.R. Sweet	M.W.K. Goh P168	*Stauroglottis*	5
Ph. celebensis H.R. Sweet	M.W.K. Goh P220	*Stauroglottis*	5
Ph. lindenii Loher	M.W.K. Goh P186	*Stauroglottis*	5
Ph. lindenii Loher	M.W.K. Goh P510	*Stauroglottis*	5
Ph. aphrodite Rchb.f.	M.W.K. Goh P102	*Phalaenopsis*	5
Ph. aphrodite Rchb.f.	M.W.K. Goh P102a	*Phalaenopsis*	5
Ph. philippinensis Golamco ex Fowlie & C.Z. Tang	M.W.K. Goh P357	*Phalaenopsis*	5
Ph. philippinensis Golamco ex Fowlie & C.Z. Tang	M.W.K. Goh P538	*Phalaenopsis*	5
Ph. philippinensis Golamco ex Fowlie & C.Z. Tang	M.W.K. Goh P537	*Phalaenopsis*	5
Ph. stuartiana Rchb.f.	M.W.K. Goh P83	*Phalaenopsis*	5
Ph. stuartiana Rchb.f.	M.W.K. Goh P225	*Phalaenopsis*	5

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Table 1. (*Continued*)

Taxon name	Accession no.	Classification (Section/subgenus)	Cluster no.
Ph. stuartiana Rchb.f.	M.W.K. Goh P229	*Phalaenopsis*	5
Ph. deliciosa Rchb.f.[a]	M.W.K. Goh P21	*Deliciosae*	5
Ph. deliciosa Rchb.f.[a]	M.W.K. Goh P520	*Deliciosae*	5
Ph. deliciosa Rchb.f.[a]	M.W.K. Goh P435	*Deliciosae*	5
Ph. pulcherrima (Lindl.) J.J. Sm.[b]	M.W.K. Goh P12	*Esmeralda*	5
Ph. pulcherrima (Lindl.) J.J. Sm.[b]	M.W.K. Goh P17	*Esmeralda*	5
Ph. pulcherrima (Lindl.) J.J. Sm.[b]	M.W.K. Goh P278	*Esmeralda*	5
Genus *Paraphalaenopsis*			
P. serpentilingua (J.J. Sm.) A.D. Hawkes	M.W.K. Goh P52		6
P. serpentilingua (J.J. Sm.) A.D. Hawkes	M.W.K. Goh P53		6
P. serpentilingua (J.J. Sm.) A.D. Hawkes	M.W.K. Goh P135		6
P. denevei (J.J. Sm.) A.D. Hawkes	M.W.K. Goh P75		6
P. denevei (J.J. Sm.) A.D. Hawkes	M.W.K. Goh P185		6
P. denevei (J.J. Sm.) A.D. Hawkes	M.W.K. Goh P489		6
P. laycockii (M.R. Hend.) A.D. Hawkes	M.W.K. Goh P196		6
P. laycockii (M.R. Hend.) A.D. Hawkes	M.W.K. Goh P488		6
P. laycockii (M.R. Hend.) A.D. Hawkes	M.W.K. Goh P198		6
P. labukensis Shim, A.L. Lamb & C.L. Chan	M.W.K. Goh P69		6
P. labukensis Shim, A.L. Lamb & C.L. Chan	M.W.K. Goh P521		6
P. labukensis Shim, A.L. Lamb & C.L. Chan	M.W.K. Goh P228		6
Ph. chibae Yukawa[a]	M.W.K. Goh P485a	*Deliciosae*	7
Ph. chibae Yukawa[a]	M.W.K. Goh P566	*Deliciosae*	7
Ph. chibae Yukawa[a]	M.W.K. Goh P568	*Deliciosae*	7

[a]Denotes plants previously classified in genus *Kingidium*.
[b]Denotes plants previously classified in genus *Doritis*.

DNA extraction

Genomic DNA was extracted from fresh leaves or flowers using Plant DNAzol® Reagent (GIBCOBRL, Life Technologies™ NY, USA) following the manufacturer's protocol. We have slightly modified the method by incorporating an additional step of extraction with phenol:chloroform (1:1, v/v), after the first phenol:chloroform step. The DNA pellets were air dried and subsequently dissolved in 50 μl TE buffer. The DNA quality was verified by 0.8% agarose gel electrophoresis (Sambrook et al., 1989).

PCR reactions

To optimize the PCR amplification conditions, experiments were carried out with varying concentrations of $MgCl_2$ (2, 4, 6 and 8 mM) and DNA template (50, 100, 150 and 200 ng/μl). The dNTPs (0.4 mM) and primers (1 μM) were used as optimized for the *Ixora* RAPD assay in our laboratory (Rajasegar et al., 1997). Twenty different 10-mer primers (Operon Technologies, Inc., Alameda, CA) were initially screened. Six of these (Table 2) were chosen for the final analysis, as they were consistent in generating products in duplicate reactions.

The polymerase chain reaction (PCR) mixture (50 μl) consisted of 50 ng of DNA template, 4 mM $MgCl_2$, 10× PCR buffer without $MgCl_2$, 0.4 mM dNTPs, 1 μM of primers and 0.5 units of *Thermus aquaticus* DNA polymerase (MBI Fermentas, Lithuania). Included with each assay was a negative control tube in which genomic DNA was omitted from the PCR reaction mixture.

The PCR reaction was carried out in a Programmable Thermal Controller, PTC-100™ (MJ

Table 2. Details of the 10-mer random primers (Operon Technologies, Inc., USA) screened for this study and the number of RAPD bands generated by the six primers for the 149 accessions of *Phalaenopsis* and *Paraphalaenopsis* species studied

Primer	Sequence (5′–3′)	%G + C	Melting temperature (Tm) (°C)	No. of polymorphic bands	Total no. of bands
OPU-01	ACGGACGTCA	60	32	–	
OPU-02	CTGAGGTCTC	60	32	–	
OPU-03[a]	CTATGCCGAC	60	32	20	20
OPU-04	ACCTTCGGAC	60	32	–	
OPU-05	TTGGCGGCCT	70	34	–	
OPU-06	ACCTTTGCGG	60	32	–	
OPU-07	CCTGCTGATC	60	32	–	
OPU-08[a]	GGCGAAGGTT	60	32	22	42
OPU-09	CCACATCGGT	60	32	–	
OPU-10[a]	ACCTCGGCAC	70	34	22	64
OPU-11	AGACCCAGAG	60	32	–	
OPU-12[a]	TCACCAGCCA	60	32	17	81
OPU-13[a]	GGCTGGTTCC	70	34	24	105
OPU-14	TGGGTCCCTC	70	34	–	
OPU-15	ACGGGCCAGT	70	34	–	
OPU-16[a]	CTGCGCTGGA	70	34	18	123
OPU-17	ACCTGGGGAG	70	34	–	
OPU-18	GAGGTCCACA	60	32	–	
OPU-19	GTCAGTGCGG	70	34	–	
OPU-20	ACAGCCCCCA	70	34	–	

[a]Denotes the six primers chosen for the study.

Research, Inc., USA). The thermal cycler was programmed to have an intitial denaturation at 95 °C for 10 min and a cycling profile of 1 min denaturation at 95 °C, 1 min annealing at 35 °C and 2 min extension at 72 °C for a total of 45 cycles, using the fastest possible transitions between each temperature. A final extension at 72 °C for 10 min was included after the last cycle.

The amplified PCR products were fractionated on a 2.0% (w/v) agarose gel in 1 × TAE buffer. Ten microliters of amplified DNA (PCR products) were loaded into each well on the agarose gel together with 2 μl of 6 × DNA loading buffer. The marker used was a mixture of equal concentrations of λHindIII and ϕ × 174 Hae III (MBI Fermentas, Lithuania).

After electrophoresis, prior to visualization of the banding profile, the gel was stained with 0.5 mg/l ethidium bromide for 5 min, destained in autoclaved water for 1 min, viewed under UV light and photographed. Bands on the photos were then scored using Gel-Pro® Analyzer version 3.0 for Windows™ (Media Cybernetics, Silver Spring MD, USA).

Cluster analysis

Only clear RAPD bands that were reproducible in at least two independent experiments, between 310 and 4361 bp, were scored numerically as present (1) or absent (0). Smeared and weak bands were excluded. The binary data obtained was then analysed with the programme NTSYSpc version 2.1 (Rohlf, 2000). The SIMQUAL module was first used to generate a similarity matrix using Dice's coefficient of similarity measure as in Nei & Li (1979). The similarity matrix was converted to genetic distances using the formula $GD_{AB} = -\ln(S_{AB})$, where S_{AB} is the measure of genetic similarity between accessions A and B and is defined as: $S_{AB} = 2N_{AB}/(N_A + N_B)$, where N_{AB} is the number of bands shared by individuals A and B, and N_A and N_B are the number of bands in accessions A and B, respectively (Nei & Li, 1979). The distance matrix was then used for cluster analysis, and the SAHN module was used to produce a dendrogram with the unweighted pair-group

method with arithmetic mean (UPGMA) clustering strategy.

Results

Sources of DNA

Newly opened flowers were used as the source of DNA for RAPD analysis of most accessions. Flowers were preferred over leaves because they are more likely to be free of algae or fungi that could be found on the leaves of plants, especially in those from wild specimens. Furthermore, removing a leaf or part of a leaf from a *Phalaenopsis* plant is more harmful to the plant than if just a flower was removed since the biomass of the plant would be reduced drastically as it has on average only three to six leaves. In order to test the suitability of DNA from flowers for RAPD analysis, we subjected DNA from flowers and from leaves to RAPD analysis (data not shown). The PCR products that were obtained from leaves and flowers were similar, which indicated that DNA from the flowers is suitable for conducting such studies.

Genetic similarity

Optimization of RAPD protocols and careful selection of primers ensured that the RAPD profiles produced were reproducible. Among the 20 random primers (Table 2) used for the initial screening, six provided optimal and reproducible RAPD profiles for all the species studied. One primer, OPU4 failed to amplify any bands in all taxa. Hence, the six useful primers were used for the final analysis. A total of 123 polymorphic bands were generated using the six primers chosen, i.e., an average of 21 bands per primer. No monomorphic bands were obtained. The amplified products varied between 350 and 2500 bp. In all accessions, intraspecific similarity was higher than interspecific similarity. Among all the accessions, the range of similarity coefficients was from 0.232 to 0.992. Between accession, *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. Representative RAPD profiles for some of the taxa studied are shown in Figure 1.

Multivariate analysis

From our binary data matrix of 149 accessions, a dendrogram was obtained (Figure 2). Based on the dendrogram, seven main clusters were obtained. Following the classification of Christenson (2001), cluster 1 contains the subgenera *Aphyllae*, *Parishianae* and *Proboscidioides*. Cluster 2 contains the subgenus *Polychilos*, sections *Fuscatae* and *Polychilos*. Cluster 3 contains subgenus *Polychilos*, sections *Amboinenses* and *Zebrinae*. Cluster 4 contains three species, viz., *Ph. doweryensis*, *Ph. gigantea* and *Ph. maculata*. Cluster 5 contains all the sections in subgenus *Phalaenopsis*. Cluster 6 contains the genus *Paraphalaenopsis*. Cluster 7 consists of only one species, *Ph. chibae*.

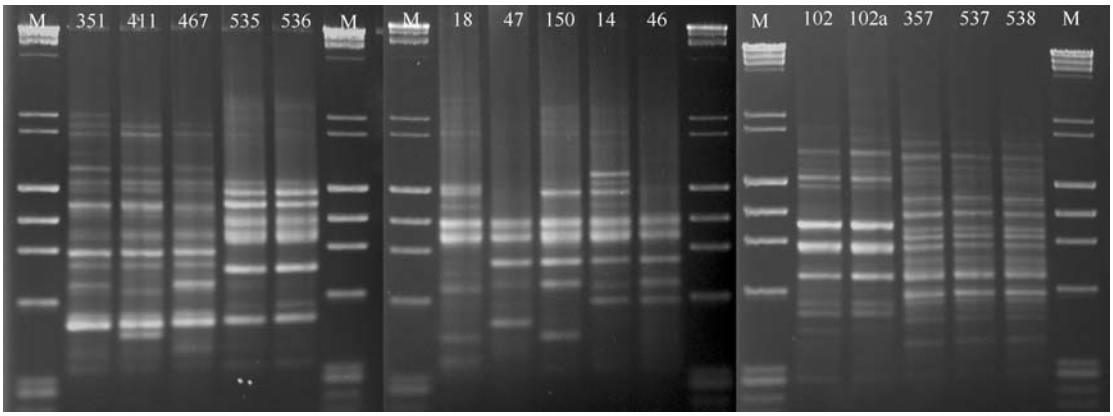

Figure 1. Representative agarose gel photographs of the PCR products obtained using OPU 16. M = λHindIII and $\phi \times 174$ Hae III marker (MBI Fermentas, Lithuania). The lane numbers given represent the accessions of plants that were used. Please refer to Table 1 for the sample identities.

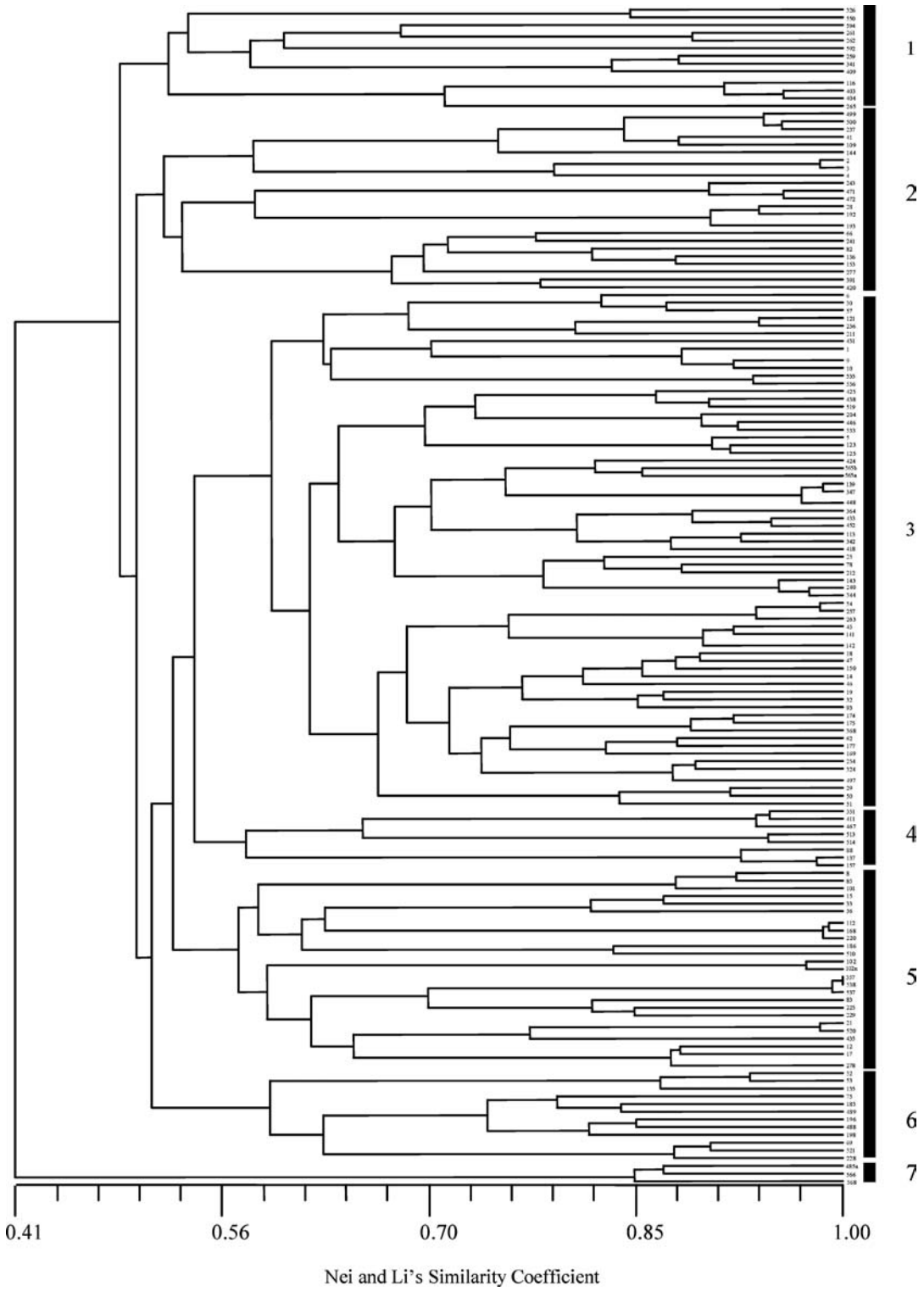

Figure 2. Dendrogram of 149 *Phalaenopsis* and *Paraphalaenopsis* accessions resulting from a UPGMA cluster analysis based on genetic distances of Nei & Li (1979) obtained from the six RAPD primers. Numbers beside the bold bars indicate the cluster number. Numbers at the end of the dendrogram branches represent the accession numbers of plants used in this study.

Discussion

In general, our RAPD data are in agreement with the classifications based on morphological characters proposed by Sweet (1980) and Christenson (2001), respectively. The limited number of RAPD primers used in our analysis generated sufficient variability to differentiate the different subgenera and even species of *Phalaenopsis*.

Taxonomic issues addressed

The latest species to be described in the genus, *Ph. doweryensis* has been thought by some workers to be a hybrid between *Ph. gigantea* and possibly another species of the section *Fuscatae* due to its morphological resemblance to its putative parent plants. Furthermore, a photograph recently published in the Orchid Digest volume 66(4), of a hybrid between *Ph. fuscata* and *Ph. gigantea*, resembles *Ph. doweryensis* morphologically. Using RAPD analysis, we were able to conclude that *Ph. doweryensis* is not an F1 hybrid. An F1 hybrid should display a RAPD profile intermediate to those of the parent plants. However, RAPD profiles of *Ph. doweryensis* are not intermediate between that of *Ph. gigantea* and other species in section *Fuscatae* (figure not shown). The clustering strategy also did not place *Ph. doweryensis* near section *Fuscatae*. *Ph. doweryensis* is however very similar to *Ph. gigantea* as observed from their RAPD profiles. This situation can arise if the hybrid is back-crossed once or twice with one of the parents, perhaps *Ph. gigantea*. Hence it is not surprising that the two were clustered together on Cluster 4 of the dendrogram (Figure 2).

RAPD markers showed that species that were previously classified under the genera *Doritis* and *Kingidium* are clustered together with species of *Phalaenopsis*. For example, both *Ph. pulcherrima* (=*D. pulcherrima*) and *Ph. deliciosa* (=*K. deliciosa*) are found in Cluster 5 together with species found in sections *Phalaenopsis* and *Stauroglottis*. Both species are also clustered next to each other in the dendrogram. *Ph. taenialis* (=*K. taenialis*) and *Ph. minus* (=*K. minus*) are found in Cluster 1.

Traditionally, *Ph. Lowii* (subgenus *Proboscidioides*) is thought to be unique in the genus and hence placed in a separate section/subgenus by both Sweet (1980) and Christenson (2001). RAPD analysis seems to have disproven this point as subgenus *Proboscidioides* was found in Cluster 1 together with subgenera *Aphyllae* and *Parishianae*.

Ph. chibae is, surprisingly, found to be the most distantly related to all other *Phalaenopsis* species and is placed in Cluster 7. Furthermore, it appears too that *Paraphalaenopsis* species are more closely related to all the *Phalaenopsis* compared to *Ph. chibae*. *Ph. chibae* is morphologically very different from the rest of the *Phalaenopsis* species because it has a transverse callus on the labellum. Otherwise, it most closely resembles *Ph. delicosa* in terms of habit and floral morphology. *Paraphalaenopsis* does not breed well with *Phalaenopsis* (Sweet, 1980). Hence, it would be most interesting to find out if *Ph. chibae* is able to breed with other species of *Phalaenopsis*, given its distant relationship with them.

RAPD data placed *Ph. micholitzii* in section *Amboinenses* (Cluster 3) whereas chloroplast sequencing data had placed it next to section *Polychilos* (Cluster 2) with high bootstrap support (data to be published elsewhere). Based on morphology, both Sweet (1980) and Christenson (2001) have placed this taxon in section *Amboinenses*. The discrepancy between classifications derived from the chloroplast sequence and RAPD data (based on the chloroplast, mitochondrial and nuclear DNA) are most probably because chloroplast and the total genomes reflect different genealogies.

Ph. cornucervi is widespread in distribution and is, in the view of Christenson (2001), phenotypically highly variable. In our study, *Ph. cornucervi* accessions from Thailand are clustered together but not placed 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.

From the dendrogram, we observed that RAPD markers are able to distinguish taxa up to the specific level for most of the *Phalaenopsis* accessions and all of the taxa for the *Paraphalaenopsis* accessions. This is evident from Clusters 1, 3–7, in which accessions of the same species were clustered together. In Cluster 2, we were only able to separate accessions up to subgeneric level as one accession of *Ph. cochlearis* was found nested between the *Ph. kunstleri* accessions.

Out of the seven clusters obtained, only two of our clusters correspond to work done by Fu et al. (1997) or/and 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*.

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, e.g., in Cluster 2 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. Therefore, 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.

Cluster analysis separated *Ph. doweryensis*, *Ph. gigantea* and *Ph. maculata* from the rest of the taxa found in section *Amboinenses*. However, Sweet (1980) and Christenson (2001) placed all three taxa in section *Amboinenses* based on the morphology of the flowers. *Ph. doweryensis* and *Ph. gigantea* are similar to each other because they have the largest leaves in the genus and inflorescences that are concealed behind the leaves. Comparatively, *Ph. maculata* is at the other extreme because it has one of the smallest plant habit in the genus. The only character that it shares with *Ph. doweryensis* and *Ph. gigantea* is the glabrous lip of the flower.

Limitations of RAPD analysis

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). Different thermal cyclers (Edwards, 1998), temperature profiles, brand of DNA polymerases (Schierwater & Ender, 1993) and the concentration of $MgCl_2$, primer and template DNA can affect the reproducibility of the RAPD assay (Muralidharan & 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 & Gale, 1992).

Conclusions

Our study clearly demonstrated that RAPD markers are useful in unambiguous separation of the genus into seven clusters and is hence a useful tool for identifying *Phalaenopsis* orchids up to the specific and/or subgeneric levels. Species previously classified under genera *Doritis* and *Kingidium* are clustered with species of *Phalaenopsis*. 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. RAPD remains a very powerful tool for this group of plants.

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