Genetic Relationship among *Paphiopedilum* Subgenus *Brachypetalum* Section Brachypetalum Using HAT-RAPD Markers

Teepaka Meesangiem, Somchit Damrianant and Theerachai Thanananta

Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Centre, Khlong Nueng, Khlong Luang, Pathum Thani 12120

Narumol Thanananta*

Faculty of Science and Technology, Valaya Alongkorn Rajabhat University under Royal Patronage, Khlong Nueng, Khlong Luang, Pathum Thani 13180

Abstract

Genetic relationship of lady's slipper orchids, *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum, 14 varieties found in Thailand, was demonstrated by HAT-RAPD markers. The total of 72 random primers were screened and 29 primers could be used for DNA amplification. Twenty-two primers were selected and used to analyze each DNA species of all samples. A dendrogram was constructed, based on polymorphic bands, and showed genetic similarities among 4 clusters with the similarity coefficients ranging from 0.32 to 0.81 as well as the analysis of genetic diversity.

Keywords: *Paphiopedilum*; genetic relationship; HAT-RAPD marker

1. Introduction

Orchid is a monocotyledon plants in the family Orchidaceae. The orchids are one of the most popular cut flower plants that widely used in domestic and aboard countries, because of colorful flowers, many shapes of flower and diverse varieties. Moreover, orchids have a long lifespan (Netnaree, 2006). *Paphiopedilum* often called the Venus slipper, its natural habitat is subtropical or tropical moist lowland forests especially in Southeast Asia. The genus *Paphiopedilum* has been divided into 7 sub-genus namely *Parvisepalum*, *Brachypetalum*, *Paphiopedilum*, *Sigmatopetalum*, *Polyantha*, *Megastaminodium* and *Cochlopetalum*. In Thailand, only subgenus *Paphiopedilum* was found, 18 species out of total 137 species worldwide (Chiramongkolgarn, 2010). Besides, stilbenes, a group of cytotoxic substances isolated from the roots of *Paphiopedilum*, was reported to be against human lung cancer (Lertnitikul *et al*., 2016), anti- inflammatory, antimicrobial, antifungal and antiproliferative activities (Naphatsawan *et al*., 2016). Nowadays, *Paphiopedilum* spp. are one of endangered orchid species in the world because of

*Corresponding author: narumolpla@yahoo.com
DOI: 10.14456/tjst.2018.2

deforestation and commercial smuggling. In addition, the morphological identification is quite difficult because of their high diversity of flowers with homologous morphology of other parts. Among the seeds of *Paphiopedilum* species, their appearances are very similar and difficult to differentiate. Hence, morphological identification is a time cost and requires skilled labors (Guo *et al*., 2016). Therefore, molecular marker can be used to solve these problems.

Molecular markers have been applied for species identification and assessment of genetic relationship. DNA markers provide a powerful tool for genetic relationship study in many organisms. There are several DNA markers based on PCR techniques (PCR-based DNA fingerprinting) including inter simple sequence repeats (ISSR) (Zhang *et al*., 2010), random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) and high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) (Thanananta *et al*., 2103). The HAT-RAPD markers are from RAPD markers by increasing the annealing temperature from 35-38 to 46 degree Celsius. These markers have been used extensively for DNA fingerprinting to classify and assess genetic relationship. The advantages of the HAT-RAPD markers are simplicity, low cost, no needed information of sequences and no influence by environmental factors (Peyachoknagul, 2009).

In the present study, HAT-RAPD markers have been employed to study genetic relationship assessment and identification of 14 *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum species, followed by constructing dendrogram, which will be conducted to a plan for orchids conservation and breeding program in the future.

2. Materials and methods

2.1 Plant materials

The plant samples in this study were 14 species of *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum. They were all found in Thailand, i.e. (1) *P. bellatulum*, (2) *P. concolor* subsp. *Reynieri*, (3) *P. concolor* var. Tonkinense, (4) *P. concolor* var. Concolor, (5) *P. concolor* subsp. *Hennisianum,* (6) *P. concolor* var. Longipetalum, (7) *P. godefroyae*, (8) *P. godefroyae* var. Leucochilum (yellow), (9) *P. godefroyae* var. Leucochilum (white), (10) *P. godefroyae* var. Ang-thong, (11) *P. niveum*, (12) *P. thaianum,* (13) *P. greyi* and (14) *P. concolor* var. Chlorophyllum.

2.2 DNA extraction

The genomic DNA was extracted from fresh leaves following the modified CTAB (cetyl trimethyl ammonium bromide procedure of Doyle and Doyle (1987), described by Thanananta *et al.* (2012). The DNA was quantified by the UV absorbance at 260 nm while the DNA quality was evaluated by 0.8 % agarose gel electrophoresis. Working solutions of the DNA were prepared by diluting the stocks at 100 ng in sterile distilled water (Sambrook *et al*., 1989)

2.3 HAT-RAPD Analysis

Seventy-two random primers (Wako Company, Japan) were tested for PCR amplification. These 12-mer length primers were used

in HAT- RAPD to investigate polymorphism extent and to select primers those are capable to create DNA fingerprinting of *Paphiopedilum*. Only the primers that produced identical polymorphic bands three times were selected for analysis. The HAT- RAPD amplification was performed in a total volume of 20 µl that contained: 100 ng of template DNA, 1x buffer (50 mM KCl, 10 mM Tris- HCl pH 9.1, 0.1 % Triton™ X-100 and 0.25 mM $MgCl_2$), 2 mM each of dATP, dGTP, dCTP and dTTP, 250 nM primer and enzyme *Taq* DNA polymerase (Vivantis, Vivantis technologies Sdn. Bhd., Malaysia) 1 unit. Amplification was performed in a thermal cycler machine (Perkin Elmer; Gene Amp PCR system 2400) as follows: an initial denaturation at 95 ºC for 3 min, cycle denaturation at 94 ºC for 30 sec, annealing at 46 ºC for 30 sec, initial extension at 72 ºC for 1 min for 40 cycles with final extension at 72 ºC for 5 min (Thanananta *et al.*, 2012). After that, the amplified products were resolved in 1.5 % agarose gel electrophoresis.

2.4 Data analysis

DNA fingerprints of HAT- RAPD were scored visually for presence (1) or absence (0) of bands for all *Paphiopedilum*. These binary data were used for calculating similarity coefficient. Then, similarity coefficient values were used to construct a dendrogram using the method of unweighted pair group with arithmetic averages (UPGMA) together with the software NTSYS-pc version 2.01e (Rohlf, 2002).

3. Result and Discussion

HAT- RAPD markers were applied to examine the genetic relationship among 14 varieties of *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum those found in Thailand. The total 72 random primers were screened and 29 primers (40.28 %) could be used for DNA amplification. Twenty-two primers were selected and used to analyze each DNA species of *Paphiopedilum*. These primers are A22, A24, A30, A32, B22, B23, B25, B27, B32, C21, C22, C28, C29, C31, D23, E23, E24, E31, E32, F23, F29 and F31. The total of 293 bands with ranging in size from 300 to 2,900 base pairs, 275 (94 %) being polymorphic bands and 18 (6 %) being monomorphic bands. Eleven of 22 random primers were able to identify each cultivars even though using only one primer. They were A24, B22, B23, B27, C21, C23, C28, C29, C31, E31 and F29 primers. An example of the polymorphisms detected with primers C29 were shown 100 % polymorphic pattern. The 18 total bands were characterized based on size with a range of 400-2,000 base pairs (Figure 1).

The estimation of genetic relationship were obtained from HAT- RAPD marker data using similarity coefficient. Pairwise coefficients of similarity for all samples are shown in Figure 2. Similarity values ranged from 0.32 (between *P. bellatulum* and *P. godefroyae*) to 0.81 (between *P. bellatulum* and *P. concolor* subsp. *Reynieri*). The results from the UPGMA cluster analysis are shown as a dendrogram in Figure 3. The dendrogram at similarity coefficients 0.57 showed a clear distinction among 14 varieties and classified them into 4 clusters. The first cluster

comprised of 5 species: *P. bellatulum*, *P. concolor* subsp. *Reynieri*, *P. concolor* var. Tonkinense, *P. concolor* subsp. *Hennisianum* and *P. concolor*. The second cluster included 2 species: *P. concolor* var. Longipetalum and *P. concolor* var. Chlorophyllum. The third cluster was composed of 6 species: *P. godefroyae*, *P. godefroyae* var. Leucochilum (yellow) , *P. godefroyae* var. Leucochilum (white), *P. niveum*, *P. thaianum* and *P. godefroyae* var. Ang-thong. The fourth cluster was only *P.* Greyi. These groups were identified by *Paphiopedilum* cultivars and morphological characteristics.

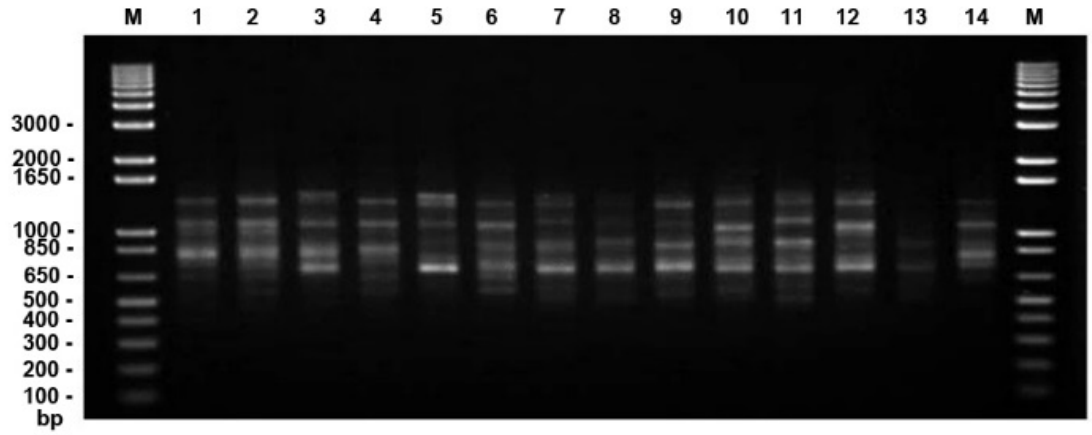

Figure 1 DNA fingerprinting of *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum using HAT-RAPD by C29 primer (TCTGCTGACCGG) [Lane M: DNA marker 1 Kb Plus DNA Ladder (Invitrogen™ Life Technology, USA), Lane 1-14: 14 species of *Paphiopedilum* section Brachypetalum.]

The results of dendrogram revealed that the closest relationship at approximately 81 % similarity occurred between *P. bellatulum* and *P. concolor* subsp. *Reynieri*, which is consistent with Wanichananan (2000), that reported species relationship in the section Brachypetalum of *Paphiopedilum* using RAPD (random amplified polymorphic DNA) Technique. Furthermore, nuclear rDNA ITS sequences showed that *P. bellatulum* and *P. concolor* subsp. *Reynieri* are closely related (Cox *et al*., 1997). In contrast, *P.* Greyi was separated from other species with low percentage of average similarity. *P. niveum* and *P. thaianum* were more closely related than *P. godefroyae*, corresponding to the research of Pumikong *et al.* (2011), who worked with these three same species in some locations of southern Thailand using RAPD markers. This research has shown a high genetic correlation between *P. thaianum* and *P. niveum*. However, *P. niveum P. thaianum* and *P. godefroyae* were classified in the same cluster.

	P. bellatulum	*P. concolor* subsp. *reynieri*	*P. concolor* var. Tonkinense	*P. concolor* var. Concolor	*P. concolor* subsp. *hennisianum*	*P. concolor* var. Longipetalum	*P. godefroyae*	*P. godefroyae* var. Leucochilum (yellow)	*P. godefroyae* var. Leucochilum (white)	*P. godefroyae* var. Ang-thong	*P. niveum*	*P. thaianum*	*P. Greyi*	*P. concolor* var. Chlorophyllum
P. bellatulum	1.00													
P. concolor subsp. *reynieri*	0.81	1.00												
P. concolor var. Tonkinense	0.65	0.62	1.00											
P. concolor var. Concolor	0.53	0.52	0.49	1.00										
P. concolor subsp. *hennisianum*	0.56	0.55	0.64	0.56	1.00									
P. concolor var. Longipetalum	0.41	0.42	0.47	0.52	0.49	1.00								
P. godefroyae	0.32	0.35	0.41	0.45	0.49	0.59	1.00							
P. godefroyae var. Leucochilum (yellow)	0.39	0.38	0.41	0.36	0.46	0.54	0.65	1.00						
P. godefroyae var. Leucochilum (white)	0.42	0.38	0.40	0.44	0.43	0.55	0.56	0.57	1.00					
P. godefroyae var. Ang-thong	0.39	0.38	0.38	0.44	0.46	0.56	0.56	0.56	0.68	1.00				
P. niveum	0.47	0.46	0.48	0.46	0.46	0.50	0.52	0.55	0.58	0.62	1.00			
P. thaianum	0.40	0.39	0.41	0.41	0.42	0.53	0.52	0.49	0.59	0.59	0.63	1.00		
P. Greyi	0.37	0.35	0.40	0.33	0.40	0.44	0.49	0.56	0.47	0.48	0.49	0.47	1.00	
P. concolor var. Chlorophyllum	0.52	0.51	0.44	0.52	0.45	0.48	0.43	0.45	0.48	0.44	0.48	0.48	0.48	1.00

Figure 2 Genetic similarity coefficient matrix for 14 individuals of *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum by HAT-RAPD.

The result in this study clearly indicated that HAT-RAPD markers have been successfully revealed relationship and classification of the *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum. The HAT-RAPD markers can be used to classify orchids such as *Dendrobium* spp. (Thomsopa *et al.*, 2014), *Rhynchostylis gigantean* (Thanananta *et al.*, 2013) and Vanda (Tongsom *et al.*, 2015). Moreover, they could be practiced with other markers for increasing an accuracy of results. For example, identification and genetic relationship analysis of *Bulbophyllum* using HAT-RAPD and ISSR markers (Saetai *et al.*, 2014), which was found that the analysis with HAT-RAPD markers and ISSR markers provided the best results. However, Tansa-nga *et al.* (2014) showed that HAT-RAPD markers were more effective than ISSR markers for genetic relationship and the discrimination of *Aerides* varieties. Likewise, Sumrith *et al.* (2014) demonstrated that HAT-RAPD gave more reliable result than ISSR markers.

4. Conclusion

The amplification of 14 cultivars of *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum using 22 primers for HAT-RAPD marker, produced 293 bands with ranging in size

from 300 to 2,900 base pairs, which 275 (94 %) being polymorphic bands and 18 (6 %) being monomorphic bands. Eleven primers could identify each cultivars even though using only one primer. A dendrogram constructed using the UPGMA method together with program NTSYS-pc version 2.01e showed that all 14 cultivars were divided in to four clusters with 0.57 of the similarity coefficient. The clusters identified by *Paphiopedilum* cultivars and extent related with their morphological. Thereby, the HAT-RAPD markers have been suggested to be a practical tool for genetic relationships study among *Paphiopedilum* cultivars, which the result can be conducted to planning in the breeding program in the future.

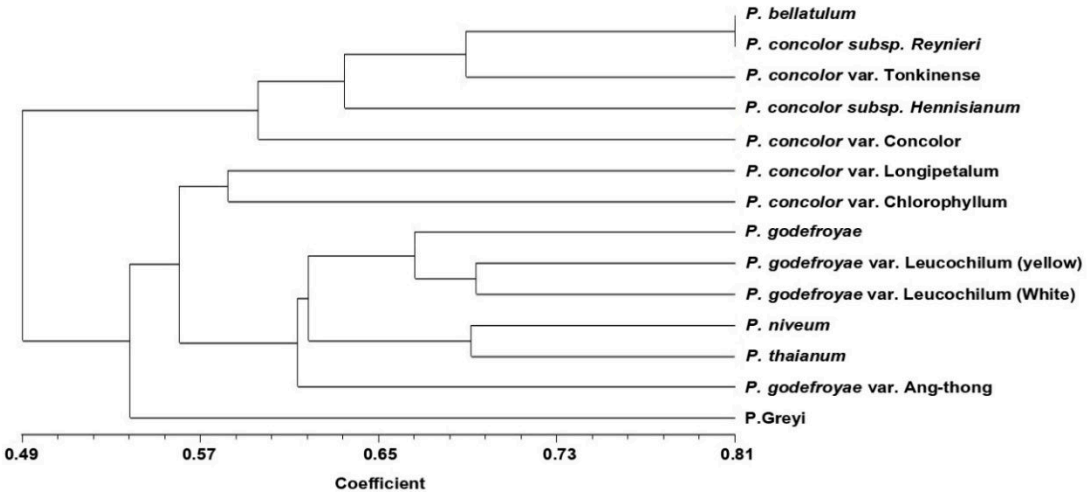

Figure 3 A dendrogram for 14 individuals of *Paphiopedilum* subgenus *Brachypetalum* section Brachypetalum generated by UPGMA cluster analysis of the genetic similarity values by HAT-RAPD.

5. References

Chiramongkolgarn, U., 2010, *Paphiopedilum*, 1st Ed, Amarin Printing & Publishing Public Company, Bangkok.

Cox, A.V., Pridgeon, A.M., Albert, V.A. and Chase, M.W., 1997, Phylogenetics of the slipper orchids (Cypripedioideae, Orchidaceae): Nuclear rDNA ITS sequences, Plant Syst. Evol. 208: 197-223.

Doyle, J.J. and Doyle, J.L., 1987, A rapid DNA isolation procedure for small quantities of fresh leaf tissue, Polym. Bull. 19: 11-15.

Guo, Y.Y, Huang, L.Q., Liu, Z.J. and Wang, X.Q., 2016, Promise and challenge of DNA barcoding in Venus slipper (*Paphiopedilum*), Plos ONE, 11(1): e0146880.

Lertnitikul, N., Jittham, P., Khankhampoch, L., Pattamadilok, C., Sukrong, S. and Suttisri, R., 2016, Cytotoxic stilbenes from the roots of *Paphiopedilum godefroyae*, J. Asian Nat. Prod. Res. 18: 1143-1150.

Naphatsawan, P., Nonthalert, L., Rutt, S. and Suchada, S., 2016, Stilbenes from *Paphiopedilum exul* roots, TJPS. 40: 116-119.

Netnaree, S., 2006, Orchids, 5th Ed, Kasetsiam-book Publications, Bangkok.

Peyachoknagul, S., 2009, DNA marker, Kasetsart University Publications, Bangkok.

Pumikong, S., Vanichanon, A. and Upho, U., 2011, Morphological charecters and genetic diversity of Lady's slipper orchids (*Paphiopedilum thaianum*, *P. niveum* and *P. godefroyae*) in some locations of southern Thailand, Faculty of Science, Thaksin University, Songkhla.

Rohlf, F.J., 2002, NTSYpc Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistics, Inc., New York.

Saetai, K., Thanananta, T. and Thanananta, N., 2014, Identification and genetic relationship analysis of *Bulbophyllum* section Sestochilus using HAT-RAPD and ISSR markers, Thai J. Sci. Technol. 3: 92-101.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, New York.

Sumrith, J., Thanananta, T. and Thanananta, N., 2014, Identification and genetic relationship analysis of colored rice cultivars using HAT-RAPD and ISSR techniques, Thai J. Sci. Technol. 3: 113-122.

Tansa-nga, W., Thanananta, T. and Thanananta, N., 2014, Identification and genetic relationship analysis of *Aerides* varieties using high annealing temperature-random amplified polymorphic DNA (HAT-RAPD) and inter-simple sequence repeat (ISSR) techniques, Thai J. Sci. Technol. 3: 102-112.

Thanananta, N., Prasit, V. and Thanananta, T., 2012, Identification of rice cultivars KDML105 and its improved cultivars by using HAT-RAPD technique, Thai J. Sci. Technol. 1: 169-179.

Thanananta, N., Pumeiam, S. and Thanananta, T., 2013, Identification and genetic relationship of *Rhynchostylis gigantean* and their hybrids using HAT-RAPD markers, Thai J. Sci. Technol. 4: 360-370.

Thomsopa, T., Thanananta, T. and Thanananta, N., 2014, Identification and genetic relationship analysis of *Dendrobium* spp., Ueang Sai group, using HAT-RAPD and ISSR techniques, Thai J. Sci. Technol. 3: 82-91.

Tongsom, J., Thanananta, T. and Thanananta, N., 2015, Genetic relationship among *Vanda* section Ascocentrum based on HAT-RAPD and ISSR, Thai J. Sci. Technol. 23: 475-484.

Wanichananan, P., 2000, A study on species relationship in the section *Brachypetalum* of *Paphiopedilum* using RAPD technique, Ph.D. Thesis, Kasetsart University, Bangkok.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.U., 1990, DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, Nucl. Acids Res. 8: 6531-6535.

Zhang, F., Lv, Y., Dong, H. and Guo, S., 2010, Analysis of genetic stability through inter simple sequence repeats molecular markers in micro propagated plantlets of *Anoectochilus formosanus* Hayata, a medicinal plant, Biol. Pharm. Bull. 33: 384-388.