P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol. 23 No.1 January - March 2018
Page. [17 - 22]

Original research article

Genetic Relationship Assessment and Identification of Strap-Leaf *Paphiopedilum* Using HAT-RAPD Markers

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Received 19 May 2017; Received in revised form 11 July 2017
Accepted 13 December 2017; Available online 21 May 2018

ABSTRACT

High annealing temperature - random amplified polymorphic DNA (HAT-RAPD) marker was used to identify and investigate the genetic relationship among 15 *Paphiopedilum* species of Venus slipper. The total of 72 primers was screened and 16 primers could be used for DNA amplification with clear amplified products to construct DNA fingerprints. The total of 248 polymorphic bands was found. A dendrogram, which constructed from the polymorphic bands using UPGMA by the NTSYS program, showed genetic similarities among 15 *Paphiopedilum* species with similarity coefficients ranging from 0.252 to 0.624. The orchids were classified into 3 clusters. These results indicated that the HAT-RAPD markers are capable to specify *Paphiopedilum*, and can be used in the breeding program and for genetic resource conservation in the future.

Keywords: *Paphiopedilum*; HAT-RAPD; orchid; identification

1. Introduction

Paphiopedilum is a popular plant for export to foreign countries. There is only one native genus *Paphiopedilum* found in Thailand, with eighteen species out of one hundred and thirty-six species worldwide. *Paphiopedilum* is a succulent plant. The leaves are striped or green color depending on the species. They are arranged alternately on two sides, short stems and colorful flowers [1]. The flower is composed of one dorsal sepal, one lateral sepal, two petals

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and one pouch which looks baggy. The origins of *Paphiopedilum* are tropical and subtropical areas of Southeast Asia [2]. Structural characteristics of *Paphiopedilum* were used for classification, such as leaves, flower and inflorescence. The genus *Paphiopedilum* can be classified into seven subgenera including *Parvisepalum, Brachypetalum, Paphiopedilum, Sigmatopetalum, Megastaminodium, Polyantha* and *Cochlopetalum* [3].

Since the characteristics of flowers and leaves are quite similar, species classification by using morphology are difficult. The molecular markers facilitate identifying species and assessment the genetic diversity of *Paphiopedilum*. DNA markers are DNA sequences with known location on a nuclear DNA, mitochondrial DNA or chloroplast DNA that can be used to identify organisms and species. It can be described as a variation that can be observed [4].

High annealing temperature - random amplified polymorphic DNA or HAT-RAPD marker is based on the single primer with 8-12 base pairs long. This marker has been developed from RAPD marker, but annealing temperature is higher than that of RAPD marker (about 46-62 °C), resulting in highly reproducible degree of polymerphism.The main advantages of HAT-RAPD marker are high number of fragments, simple method, low quantity of DNA required, low costs and no genomic information needed [5]. In this research, the researcher has studied genetic relationship assessment and identification of strap-leaf *Paphiopedilum* using HAT-RAPD markers.

2. Materials and Methods

2.1 Plant materials

A total of 15 *Paphiopedilum* species was examined. A list of *Paphiopedilum* species are provided in Table 1.

2.2 DNA extraction

The *Paphiopedilums* genomic DNA was extracted from leaves by a modification of the cetyltrimethylammonium bromide (CTAB) method Doyle and Doyle [6], and the method from Thanananta *et al.*, [7]. After that, the quantity and quality of extracted DNA were verified using the absorbance at 260 and 280 nm. DNA was separated on 0.8 % agarose gel electrophoresis and. stored at -20 °C [8].

2.3 HAT-RAPD amplification

Screening of 72 primers (Wako Company, Japan) was performed using 15 species of *Paphiopedilum*. DNA amplification was performed in a volume of 20 µl that contained: 100 ng/µl in buffer (50 mM KCl, 10 mM Tris-HCl pH 9.1, 0.1 % Triton™ X-100 and 0.25 mM $MgCl_2$), 200 µM each of dATP, dGTP, dCTP and dTTP, 250 nM of primer and 1 unit of *Taq* DNA polymerase (Vivantis technologies Sdn. Bhd., Malaysia). DNA was amplified by thermal cycler using the following cycling steps: 94 °C for 3 min, 40 cycles of denaturing at 94 °C for 30 s, annealing at 46 °C for 30 s, extension at 72 °C for 1 min, and a final extension 5 min at 72 °C, hold at 4 °C. PCR products were separated on 1.5 % agarose gel by electrophoresis [7].

Table 1 List of 15 *Paphiopedilum* species

No.	Name
1	*Paphiopedilum villosum* (Lindl.) Stein
2	*Paphiopedilum barbigerum* var. coccineum
3	*Paphiopedilum insigne* (Wallich ex Lindley) Pfitzer
4	*Paphiopedilum gratrixianum* (Mast.) Guillaumin
5	*Paphiopedilum hirsutissimum* var. esquirolei
6	*Paphiopedilum exul* (Ridl.) Rolfe
7	*Paphiopedilum rothschildianum* (Rchb.f.) Stein
8	*Paphiopedilum primulinum* M.W.Wood & P.Taylor

9	*Paphiopedilum vejvarutianum* O.Gruss & Roellke
10	*Paphiopedilum spicerianum* (Rchb.f.) Pfitz.
11	*Paphiopedilum parishii* (Rchb.f) Stein
12	*Paphiopedilum charlesworthii* (Rolfe) Pfitz.
13	*Paphiopedilum dianthum* Tang & Wang
14	*Paphiopedilum henryanum* Braem
15	*Paphiopedilum praestans* (Rchb.f.) Pfitz.

2.4 Data analysis

Comparison of fingerprint form HAT-RAPD marker in 15 species of *Paphiopedilum*. Bands from the fingerprints were counted (1) for present and (0) for absent. After that, unweighted pair group method of arithmetic averages (UPGMA) was used for cluster analysis by NTSYS-pc ver. 2.01e. [9].

3. Results and Discussion

HAT-RAPD marker was used to study for genetic relationship between species of *Paphiopedilum*. From random primers, total 72 primers were screened and 16 primers could be used for DNA amplification giving clear amplified products to construct DNA fingerprints that included A22, A29, A30, A32, B22, B23, B25, C21, C22, C28, D23, E22, E23, E32, F23 and F25. The total of 248 polymorphic bands was found. DNA fingerprints showed PCR products between 2,300 to 100 bp. An example of DNA fingerprints from primer A30 was displayed in Fig. 1. The figure showed total PCR products of 22 bands with a size ranging between 1,700 to 270 bp.

A dendrogram, which constructed based on polymorphic bands using UPGMA by the NTSYS program, showed genetic similarities among 15 *Paphiopedilum* species with similarity coefficients ranging from 0.252 to 0.624 (Fig. 2) and divided these orchids into 3 clusters. Clusters 1 included 3 subclusters. Subcluster 1.1 was comprised of *Paphiopedilum villosum* (Lindl.) Stein, *Paphiopedilum gratrixianum* (Mast.) Guillaumin, *Paphiopedilum insigne* (Wallich ex Lindley) Pfitzer and *Paphiopedilum barbigerum* var. coccineum. Subcluster 1.2 included *Paphiopedilum hirsutissimum* var. esquirolei and *Paphiopedilum exul* (Ridl.) Rolfe. Subcluster 1.3 was comprised of *Paphiopedilum Charlesworthii* (Rolfe) Pfitzer, *Paphiopedilum henryanum* Braem, *Paphiopedilum spicerianum* (Rchb. f) Pfitz and *Paphiopedilum charlesworthii* (Rolfe) Pfitzer, Clusters 2 included *Paphiopedilum rothschildianum* (Rchb.f.) Stein, *Paphiopedilum primulinum* M.W. Wood & P. Taylor, *Paphiopedilum parishii* (Rchb.f.) Stein and *Paphiopedilum dianthum* Tang & Wang. Clusters 3 contained only the *Paphiopedilum praestans* (Rchb.f.) Pfitz. (Fig. 3). HAT-RAPD marker can be used to classify *Paphiopedilum* in to 15 species.

HAT-RAPD marker showed efficacy for studying genetic relationship in *Paphiopedilum* that can divide *Paphiopedilum* species according to Chung *et al*. [10] that studied genetic relationship and differentiation of *Paphiopedilum* and *Phragmepedium* based on RAPD analysis. In addition, there are other research showed efficacy of HAT-RAPD marker for example *Curcuma* [11], rice [12], *Bulbophyllum* section Sestochilus [13], *Brassica* spp. [14], and *Coelogyne* [15]. Moreover, DNA fingerprints from HAT-RAPD markers were reproducible and high polymorphic DNA [16-18].

4. Conclusion

HAT-RAPD marker was used to study for genetic relationship between species of *Paphiopedilum*. The total 72 random primers were screened and 16

primers could be used for DNA amplification giving clear amplified products to construct DNA fingerprints. A dendrogram, which constructed based on polymorphic bands using UPGMA by the NTSYS program, showed genetic similarities among 15 *Paphiopedilum* species with similarity coefficients ranging from 0. 252 to 0.624 and divided these orchids into 3 clusters. HAT- RAPD marker can classify *Paphiopedilum* in 15 species.

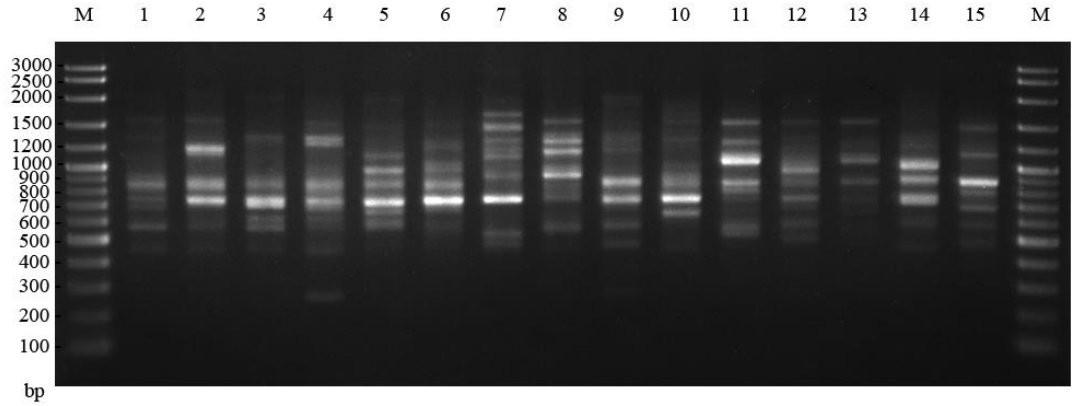


Fig. 1 HAT-RAPD DNA profiles of *Paphiopedilum* 15 species with the primers A30. [Lane M is molecular weight marker 1 Kb plus DNA Ladder (Invitrogen™ Life Technology, USA), Lanes 1-15: (1) *P. villosum* (Lindl.) Stein (2) *P. barbigerum* var. coccineum (3) *P. insigne* (Wallich ex Lindley) Pfitzer (4) *P. gratrixianum* (Mast.) Guillaumin (5) *P. hirsutissimum* var. esquirolei (6) *P. exul* (Ridl.) Rolfe (7) *P. rothschildianum* (Rchb.f.) Stein (8) *P. primulinum* M.W.Wood & P.Taylor (9) *P. vejvarutianum* O.Gruss & Roellke (10) *P. spicerianum* (Rchb.f) Pfitz. (11) *P. parishii* (Rchb.f) Stein (12) *P. charlesworthii* (Rolfe) Pfitz. (13) *P. dianthum* Tang & Wang (14) *P. henryanum* Braem (15) *P. praestans* (Rchb.f) Pfitz.]

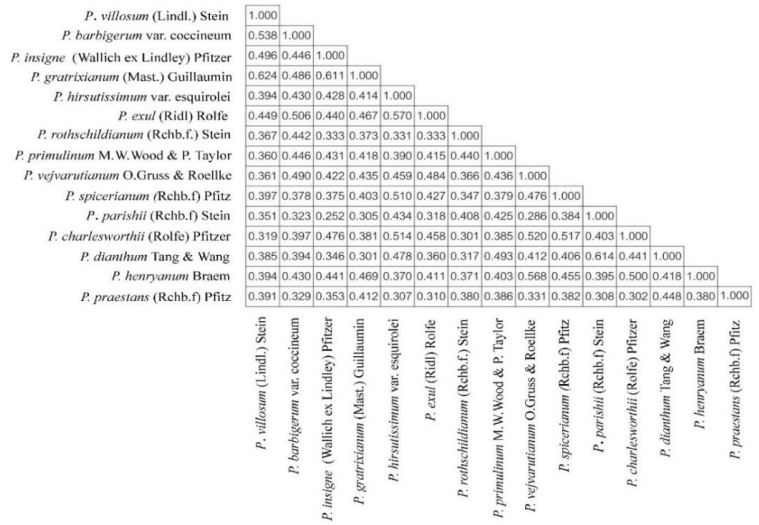
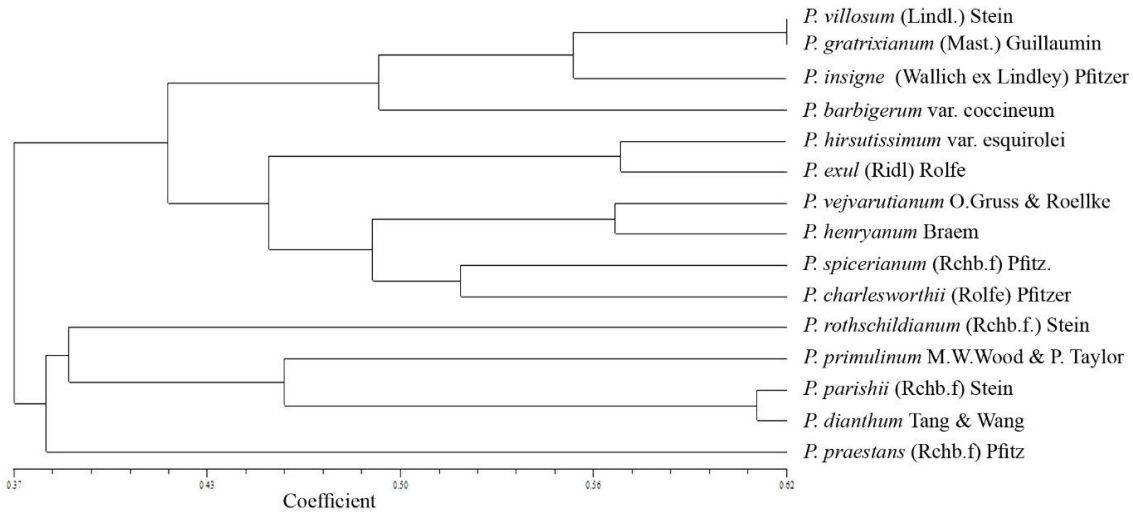


Fig. 2 This picture show similarity coefficient of *Paphiopedilum* 15 species from HAT-RAPD marker.

Fig. 3 A dendrogram of *Paphiopedilum* 15 species from HAT-RAPD marker.



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