Songklanakarin J. Sci. Technol.
39 (4), 471-477, Jul - Aug. 2017

SJST
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Original Article

RAPD fingerprinting and genetic relationship of *Gardenia* species in Thailand

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Received: 2 March 2016; Revised: 15 June 2016; Accepted: 8 July 2016

Abstract

DNA based molecular markers have a potential utility in herbal medicine analysis and widely used for studying genetic relationship of medicinal plant species. Therefore, this study aims to assess the genetic relationship among eleven *Gardenia* species collected from different locations in Thailand using random amplified polymorphic DNA (RAPD) marker. Ninety primers were initially screened, out of which 20 primers generated 579 reproducible bands of different sizes with an average of 28.95 bands per primer. The mean percentage of polymorphic bands was 99.5%. Similarity index ranged from 0.089 to 0.332. The highest similarity index (0.332) was found between *Gardenia lineata* and *G. jasminoides* while the lowest similarity index (0.089) was found between *G. carinata* and *G. sootepensis*. A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) and can be divided into 2 distinct clusters which correlated with their morphological characteristics.

Keywords: *Gardenia*, genetic relationship, RAPD analysis, DNA fingerprint

1. Introduction

Gardenia is a genus of flowering plant in the family Rubiaceae containing about 250 species, indigenous to the tropical and subtropical regions of Africa, Asia, Madagascar and Pacific islands (Suwannakud *et al.*, 2014; Tao *et al.*, 2011). Twenty-two species of *Gardenia* have been recorded, among these thirteen species are natively to Thailand (Puff *et al.*, 2005; Smittinand, 2014). The *Gardenia* species have highly medicinal values in traditional medicine as anti-cancer, anti-HIV, antitopoisomerase IIa, antiangiogenic, anti-apoptotic and thrombolytic activity (Jainul *et al.*, 2014; Kongkum *et al.*, 2013; Parmar & Sharma, 2000; Phatak, 2015; Phromnoi *et al.*, 2010; Pudhom *et al.*, 2012; Reutrakul *et al.*, 2004; Tuchinda *et al.*, 2004; Wang *et al.*, 2004).

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DNA-based markers are widely used for authentication and quality assurance of medicinal plant species due to the genetic information of each species is unique and not dependent of age, physiological conditions and environmental factors (Pourmohammad, 2013). Various DNA markers have been applied for studying the genetic relationship of medicinal plant including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), single nucleotide polymorphisms (SNPs) which each technique has their drawbacks and advantages. RAPD is one of the most frequently used method in the studies of many organisms including medicinal plants due to its rapidity, simplicity and absence of any need for prior genetic information of the plant (Chirag *et al.*, 2011; Khan *et al.*, 2009). RAPD markers have been used for evaluation of genetic diversity, molecular characterization as well as authentication of plant species such as *Urtica parviflora*

Roxb. (Chirag *et al.*, 2011), *Piper nigrum* (L.) (Khan *et al.*, 2010), *Terminalia bellirica* (Roxb.) (Bharti & Vijaya, 2013), *Phyllanthus* species (Manissorn *et al.*, 2010). RAPD has also been used to analyze genetic relationship of *Gardenia* species in both of intra-species aspect focusing on *G. jasminoides* (Mei *et al.* 2015) and inter-species aspects focusing on *G. jasminoides*, *G. taitensis* and *G. carinata* (Thanananta *et al.*, 2011). However, many species of *Gardenia* in Thailand are still lacking of genetic information. Because of the medicinal and scientific importance of *Gardenia* species, genetic information of this genus should be investigated. Despite the medicinal and scientific importance of *Gardenia* species, genetic information of this genus is still limited. Therefore, this present study aims to evaluate the genetic relationship among eleven species of *Gardenia* existing in Thailand using RAPD marker.

2. Materials and Methods

2.1 Plant materials

Fresh young leaves of eleven species of *Gardenia* namely *G. jasminoides*, *G. carinata*, *G. collinsae*, *G. griffithii*, *G. lineata*, *G. obtusifolia*, *G. sootepensis*, *G. thailandica*, *G. taitensis*, *G. tubifera* and *G. vietnamensis* were collected from different locations throughout Thailand during 2013-2014. Three individual of each eleven *Gardenia* species were collected (n = 33). All plant materials were authenticated by expert (N.R.) and voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University. *Ixora finlaysoniana* (Rubiaceae) and *Cassia timoriensis* (Caealpiniaceae) were used as out-group samples for RAPD analysis.

2.2 DNA extraction

Genomic DNA was individually extracted from the fresh young leaves of *Gardenia* species and out-group samples using DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The obtained DNA was run on 1 % agarose gel, stained with ethidium bromide and photographed under UV light (INGENIUS3, SYNGENE). The quantity and quality of DNA were estimated by measuring the absorbance at 260 nm and 280 nm using spectrophotometer (SPECORD210/PLUS, Germany). The extracted genomic DNA were diluted with 1 x TE (Tris–EDTA) buffer to make the final concentration of 10 ng/µl and stored at -20°C for DNA template in RAPD analysis.

2.3 RAPD analysis of *Gardenia* species

RAPD analysis was initially screened using 90 commercial primers (primer set of OPA-OPN from Operon Technology, USA and primer sets of RAPD, A, F from Eurofins Genomics company, USA). The amplification reaction was carried out in 20 µl reaction containing of Go*Taq* Green Master Mix (Promega), 5.0 mM Mg^{2+}, 1.5 U *Taq* DNA polymerase, 2 ng DNA template, 200 µM dNTPs and 0.8 µM primer. The PCR cycle was carried out with the initial denaturation at 94°C for 2 minutes followed by 45 cycles of 94°C for 30 s, 36°C for 2 minutes, 72°C for 2 minutes and a final extension of 72°C for 7 minutes using thermal cycler (ProFlex PCR System). The amplified fragments were separated on 1.5% agarose gel electrophoresis along with 100 bp DNA ladder and 1Kb (BioRad) as DNA markers. Gels were stained with ethidium bromide, visualized and photographed under UV light (INGENIUS3, SYNGENE).

2.4 Data analysis

RAPD bands were scored as either present (1) or absent (0) to create a binary data set and entered into a binary data matrix as discrete variable. Nei and Li (1979) similarity coefficient was calculated for all pair-wise species. A dendrogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) clustering by GeneTools and GeneDirectory software (SYNGENE).

3. Results

3.1 RAPD analysis of *Gardenia* species

The RAPD analysis of 11 *Gardenia* species (in triplicate) were initially screened with 90 arbitrarily primers. Among these, 20 primers produced 579 clear and reproducible polymorphic bands ranging from 15 to 42 bands with an average 28.95 bands per primer (Table 1). The amplified fragments varied from 193 to 3702 base pair (bp) in size. The highly percentage of polymorphism was obtained from all 20 primers (95-100%). The RAPD fingerprint of 11 *Gardenia* species obtained from OPD-07, OPF- 04, OPM-07, OPB-10 and F-25 primers was showed in Figure 1. The highest number of polymorphic bands (42) was obtained from primers OPD-07 (Figure 1A) and the lowest (15) from primers OPF-04 (Figure 1B). Monomorphic band in all *Gardenia* species was obtained from primer OPM-07 (Figure 1C) and OPB-10 (Figure 1D) while F-25 primer showed monomorphic band in all *Gardenia* species and *Ixora finlaysoniana* (out group sample in Rubiaceae Family) (Figure 1E).

3.2 Genetic relationship of 11 *Gardenia* species based on RAPD analysis

To evaluate the genetic relationship, RAPD bands produced from 20 primers were scored and a phylogenetic dendrogram was constructed between 11 *Gardenia* species (Figure 2). Dice similarity index (SI) among 11 *Gardenia* species ranged from 0.089 to 0.332 (Table 2). The highest similarity index (0.332) was found between *G. lineata* and *G. jasminoides* while the lowest similarity index (0.089) was found between *G. carinata* and *G. sootepensis*. The phylogenetic dendrogram can be divided into 2 main clusters.

Table 1. List of 20 RAPD primers and the number of amplified bands, size range and percentage of polymorphic bands in 11 *Gardenia* species.

Primer name	Primer sequence (5' to 3')	Total amplified bands	Fragment size range (bp)	Polymorphic bands	Polymorphism (%)
OPA-04	AATCGGGCTG	32	196-2658	32	100.0
OPB-04	GGACTGGAGT	18	316-1865	18	100.0
OPB-10	CTGCTGGGAC	20	255-1942	19	95.0
OPC-04	CCGGATCTAC	32	357-2327	32	100.0
OPC-06	GAACGGACTC	40	278-2569	40	100.0
OPC-08	TGGACCGGTG	34	242-2600	34	100.0
OPC-12	TGTCATCCCC	25	362-2124	25	100.0
OPC-20	ACTTCGCCAC	24	382-2309	24	100.0
OPD-07	TTGGCACGGG	42	193-2286	42	100.0
OPF-04	GGTGATCAGG	15	399-2297	15	100.0
OPF-07	CCGATATCCC	18	519-3509	18	100.0
OPL-01	GGCATGACCT	25	331-2135	25	100.0
OPL-05	ACGCAGGCAC	26	391-1803	26	100.0
OPM-07	CCGTGACTCA	30	291-2279	29	96.7
OPN-16	AAGCGACCTG	32	239-2438	32	100.0
RAPD02	TTCCGAACCC	35	287-2440	35	100.0
RAPD07	GAGGTCCAGA	36	238-2894	36	100.0
A-29	GGTTCGGGAATG	30	424-3702	30	100.0
F-25	CCAGATCCGAAT	30	482-2046	29	96.7
F-29	GCCGCTAATATG	35	411-3579	35	100.0
Total		579	193-3702	576	99.5

Table 2. Nei and Li's genetic similarity index among eleven *Gardenia* species based on RAPD markers.

Gardenia Species	*G. lineata*	*G. jasminoides*	*G. tubifera*	*G. obtusifolia*	*G. vietnamensis*	*G. taitensis*	*G. thailandica*	*G. sootepensis*	*G. griffithii*	*G. collinsae*	*G. carinata*	*I. finlaysoniana*	*C. timoriensis*
G. lineata	1												
G. jasminoides	0.332	1											
G. tubifera	0.215	0.211	1										
G. obtusifolia	0.142	0.185	0.187	1									
G. vietnamensis	0.205	0.164	0.169	0.128	1								
G. taitensis	0.148	0.121	0.187	0.205	0.208	1							
G. thailandica	0.118	0.103	0.192	0.175	0.199	0.268	1						
G. sootepensis	0.111	0.134	0.166	0.139	0.167	0.232	0.328	1					
G. griffithii	0.179	0.153	0.155	0.127	0.094	0.083	0.110	0.121	1				
G. collinsae	0.118	0.140	0.145	0.131	0.106	0.115	0.162	0.149	0.129	1			
G. carinata	0.143	0.104	0.099	0.114	0.094	0.111	0.111	0.089	0.106	0.160	1		
I. finlaysoniana	0.113	0.116	0.079	0.123	0.098	0.094	0.103	0.089	0.099	0.116	0.057	1	
C. timoriensis	0.065	0.068	0.117	0.109	0.085	0.119	0.082	0.082	0.079	0.061	0.058	0.093	1

Cluster I includes 9 *Gardenia* species (*G. jasminoides*, *G. griffithii*, *G. lineata*, *G. obtusifolia*, *G. sootepensis*, *G. thailandica*, *G. taitensis*, *G. tubifera* and *G. vietnamensis*) showing 0.083 to 0.332 similarity index and can be divided into two subgroups; subgroup 1 includes four *Gardenia* species (*G. lineata*, *G. jasminoides*, *G. tubifera*, and *G. obtusifolia*) and subgroup 2 includes five *Gardenia* species (*G. griffithii*, *G. sootepensis*, *G. thailandica*, *G. taitensis*, and *G. vietnamensis*). Cluster II includes only two *Gardenia* species (*G. carinata* and *G. collinsiae*) showing 0.089 to 0.162

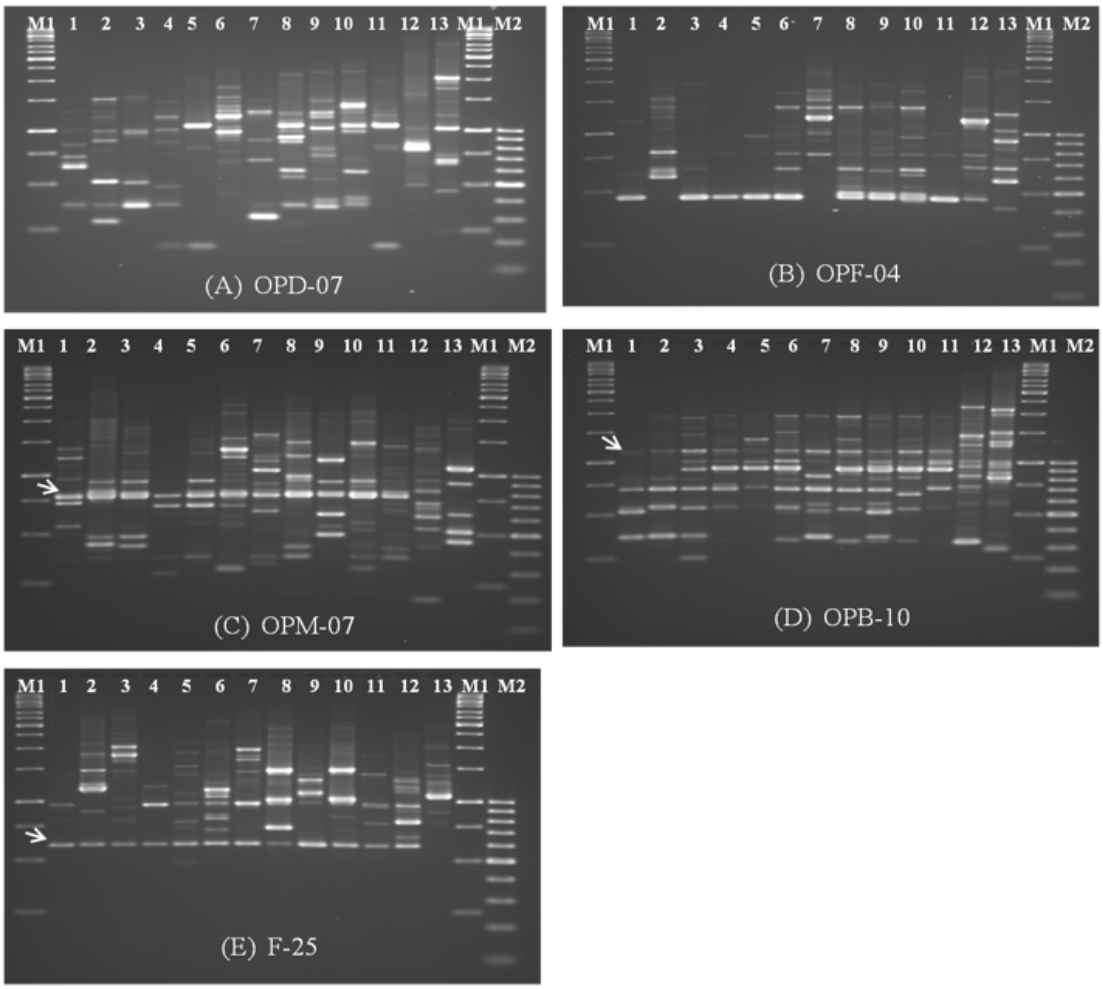

Figure 1. RAPD fingerprints of 11 *Gardenia* species obtained from (A) OPD-07, (B) OPF-04, (C) OPM-07, (D) OPB-10, and (E) F-25 primers. M1 and M2: 1 kb and 100 bp molecular weight marker respectively, lane 1 = *G. carinata*, lane 2 = *G. collinsae*, lane 3 = *G. griffithii*, lane 4 = *G. jasminoides*, lane 5 = *G. lineate*, lane 6 = *G. tubifera*, lane 7 = *G. obtusifolia*, lane 8 = *G. sootepensis*, lane 9 = *G. taitensis*, lane 10 = *G. thailandica*, lane 11 = *G. vietnamensis*, lane 12 = *Ixora finlaysoniana*, lane 13 = *Cassia timoriensis*. Arrows indicated monomorphic bands.

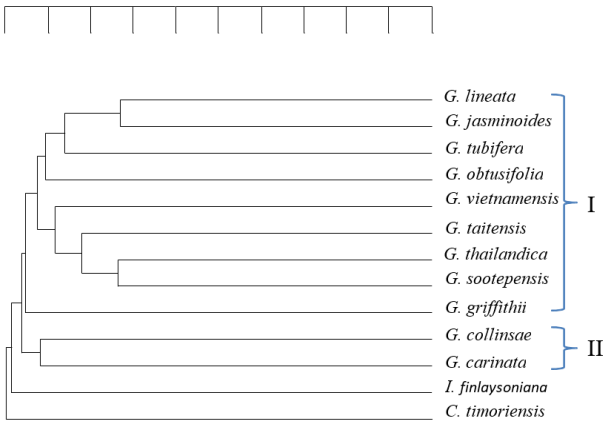

Figure 2. Genetic relationship based on UPGMA between eleven *Gardenia* species. The scale indicates the genetic similarities between individual.

similarity index. Out-group samples (*I. finlaysoniana* and *C. timoriensis*) were clearly separated from all *Gardenia* species.

Among 20 selected primers for reproducibility of RAPD results, nine primers (A29, OP B-10, OP C-04, OP C-06, OP C-08, F25, OP A-04, OP D-07and RAPD02) produced the unique bands for 7 *Gardenia* species (*G. lineata*, *G. Griffithii*, *G. obtusifolia*, *G. sootepensis*, *G. vietnamensis*, *G. taitensis*, and *G. collinsae*) as presented in Table 3.

4. Discussion

The genetic information of *Gardenia* species in Thailand is still limited. Previously reported from some studies almost focus mainly on *G. jasminoides* such as genetic characterization and authentication of *G. jasminoides* in different regions of China using RAPD analysis (Mei *et al.*, 2015), genetic diversity and biogeography of *G. jasminoides* based on AFLP markers (Han *et al.*, 2007), genetic relationships between *G. jasminoides* var. *radicans* and *G. jasminoides* for. *grandiflora* by RAPD (Huh and Choi, 2005), isolation and characterization of twenty-two polymorphic microsatellite markers from *G. jasminoides* (Xu *et al.*, 2014), comparison of *G. jasminoides* cultivars using isozymes and RAPD markers (Criley *et al.*, 2008).

In this study, RAPD analysis of 11 *Gardenia* species in Thailand including seven native species (*G. carinata*, *G. collinsiae*, *G. griffithii*, *G. obtusifolia*, *G. sootepensis*, *G. thailandica*, and *G. tubifera*) and four introduced species (*G. jasminoides*, *G. lineata*, *G. taitensis*, and *G. vietnamensis*) was carried out with 20 primers. Among seven native species, the similarity index varied from 0.089 to 0.328. The highest value was found between *G. sootepensis* and *G. thailandica* which coincide with the previous study reported among 11 *Gardenia* species (*G. carinata*, *G. collinsae*, *G. elata*, *G. jasminoides*, *G. obtusifolia*, *G. saxatilis*, *G. sootepensis*, *G. thailandica*, *G. gjellerupii*, *G. taitensis* and *G. volkensii*), the highest similarity value among native species were *G. sootepensis* and *G. thailandica* (Suwannakud *et al.*, 2014). When consider the four introduced species, the similarity index ranging from 0.121 to 0.332 and the highest similarity value was found between *G. jasminoides* and *G. lineata*. The highest similarity index between native and introduce species was found between *G. thailandica* and *G. taitensis* (0.268). The phylogenetic dendrogram based on RAPD can be divided eleven species of *Gardenia* into 2 main clusters, cluster I consisted of nine native and introduce species (*G. jasminoides*, *G. griffithii*, *G. lineata*, *G. obtusifolia*, *G. sootepensis*, *G. thailandica*, *G. taitensis*, *G. tubifera* and *G. vietnamensis*), which share their some morphological characteristics such as large size of flower, growing into tree or shrub whereas cluster II consisted of two native species (*G. collinsae* and *G. carinata*) which have small size of flower and growing into tree. In this study, 20 RAPD primers generated DNA fingerprinting of eleven *Gardenia* species which can be used as a qualitative diagnostic tool for identification of *Gardenia* species. RAPD markers has main advantages include simple, rapid, efficient, no requirement of sequence information for design of specific primers, require only small amounts of DNA template, procedure can be automated, high number of fragments, arbitrary primers are easily purchased and unit costs per assay are low compared to other marker technologies (Kumar & Gurusubramanian, 2011). However, the limitation of RAPD is the reproducibility and cannot differentiate dominant homozygote from heterozygote. To concern about reproducibility, quality and quantity of DNA template, PCR buffer, concentration of magnesium chloride, primer to template ratio and annealing temperature must be optimized. Moreover, the RAPD primer should contain minimum of 40% GC content and the absence of palidromic sequence to avoid self-annealing of primer. The present or absent of polymorphic bands due to the mismatches at the primer site, changes in DNA sequence that inhibit primer binding or the length of amplified region between primer sites. RAPD bands were considered to be polymorphic when it present in some individual but absent in others while monomorphic was presented in all the individuals. There are some specific or unique band was found in nine primers. The polymorphic banding pattern which is the specific or unique band derived from RAPD marker can be further developed as SCAR (sequence characterized amplified region) marker for rapid and simple identification of medicinal plant species.

Table 3. Unique bands for seven *Gardenia* species generated from nine RAPD primers.

Primer	*G. lineata*	*G.Griffithii*	*G. oftusforia*	*G. sootepensis*	*G. vietnamensis*	*G. taitensis*	*G. collinsae*
A29	622 bp	666 bp					
OP B-10		250 bp					
OP C-04			375 bp	357 bp			
OP C-06					449 bp		
OP C-08	317 bp	243 bp				270 bp	302 bp
F25	484 bp						
OP A-04		196 bp					
OP D-07			305 bp				282 bp
RAPD02			355 bp				

Although morphology-based identification of plant species is still the most widely used approach but it requires considerable skills and taxonomy expertise. Therefore, complementary methodologies to the conventional morphology-based identification of plant species are necessary required, especially techniques that can be used routinely providing a simple and universal application. RAPD markers can be used to identification of plant materials in many forms especially in powder form as well as in some parts of plant organs such as some part of leaf which are difficult to identified by observation only. Another valuable feature of RAPD is that, in contrast to morphology or allozyme based approaches, RAPD provide consistent markers that are physiologically independent and can be applied in species discrimination for any ontogenic stage, starting from the embryo (Costa *et al.*, 2004).

5. Conclusions

In conclusion, based on the study of eleven *Gardenia* species in Thailand using RAPD fingerprinting provides the greater information for assessment of the genetic diversity and relationships. The information obtained from this study can be used for plant identification.

Acknowledgements

This research was scholarly supported by the scholarship from College of Public Health Sciences, Chulalongkorn University, and The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund). Authors would like to thanks College of Public Health Sciences, Chulalongkorn University for their facilities.

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