LEAF CONSTANTS AND RAPD MARKER OF *Jasminum multiflorum* CULTIVARS EXISTING IN THAILAND

Jindaporn Phithaksilp[1], Nijsiri Ruangrungsi[1, 2], Kanchana Rungsihirunrat[1, *]

[1] College of Public Health Sciences Chulalongkorn University, Bangkok, 10330, Thailand
[2] Faculty of Pharmacy, Rangsit University, Pathumthani, 12000, Thailand

ABSTRACT:

Background: Medicinal plant identification is necessary for ensuring their efficacy and safety in quality control of herbal products.

Methods: Four *Jasminum multiflorum* cultivars (Maluli, Maluli Sichomphu, Maluli Phum and Mali Ngachang) existing in Thailand was first studied for their leaf constants (stomatal number, stomatal index, epidermal cell number, epidermal cell area, palisade ratio, vein termination, vein islet number and trichome number) and random amplified polymorphic DNA (RAPD) fingerprint.

Results: Leaf constants analysis demonstrated that trichome number was useful to identify these 4 *J. multiflorum* cultivars. According to the present of trichome on adaxial surface of leaf, *J. multiflorum* caltivar Maluli Phum can be distinguished. Among the rest 3 cultivars, the number of trichome on abaxial surface (17.49 ± 3.66) describes *J. multiflorum* cultivar Mali Ngachang. *J. multiflorum* cultivar Maluli and Maluli Sichomphu can be characterized by vein termination number. Leaf constants evaluation is proved to be one of the methods capable in plant authentication even among cultivar. For RAPD analysis among 4 *J. multiflorum* cultivars, dice similarity index ranged from 0.2583-0.7922. The cluster diagram could be divided into two clusters. Cluster I includes 2 cultivars of *J. multiflorum* cultivar Maluli Sichomphu and Maluli showing 0.7922 similarity index and cluster II includes 2 cultivars of *J. multiflorum* cultivar Maluli Phum and Mali Ngachang showing 0.4222 similarity index.

Conclusions: Leaf constants (stomatal number, stomatal index, epidermal cell number, epidermal cell area, palisade ratio, vein termination, vein islet number and trichome number) and RAPD fingerprints are useful techniques for plant cultivar identification.

Keywords: *Jasminum multiflorum*, Cultivar, Leaf constants, RAPD fingerprint

DOI: *Received: March 2016; Accepted: April 2016*

INTRODUCTION

The genus *Jasminum* contains more than 200 different species distributed in tropics and subtropical regions. *Jasminum multiflorum* (Burm. f.) Andr. is an evergreen flowering shrub belongs to the genus *Jasminum* in Oleaceae family [1]. The oil extracted from jasmine flower has highly value for producing of hair oil, perfume, cosmetic, flavoring tea and flavoring mount wash [2-4]. Phytochemical studies revealed that flowers of *J. multiflorum* contain cis-jasmone, eugenol, alpha-farnesene and hexyl benzoate [5]. In Thailand, there are four *J. multiflorum* cultivars (Maluli, Maluli Sichomphu, Maluli Phum and Mali Ngachang) [6] (Figure 1). Cultivar identification and estimating genetic diversity can be done by several ways which include morphological examination, microscopical evaluation and molecular analysis. Microscopic examination is a conventional method for identification of plant structural features under microscope due to its simple, rapid and inexpensive method. Leaf constant

[*] *Correspondence to: Kanchana Rungsihirunrat*
E-mail: kanchana.r@chula.ac.th

Cite this article as: Manoyos V, Tangmunkongvorakul A, Srithanaviboonchai K, Yangyuenkul S, Grimes RM. Leaf constants and RAPD marker of *Jasminum multiflorum* cultivars existing in Thailand. J Health Res. 2016; 30(5): 329-35. DOI:

(A) Maluli

(B) Maluli Sichomphu

(C) Maluli Phum

(D) Mali Ngachang

Figure 1 Four *Jasminum multiflorum* cultivars existing in Thailand

parameters have been used for medicinal plant identification [7, 8]. Recently, molecular technique has been widely used due to a robust and simply assay for determination of DNA variation in medicinal plants and crude drugs without the effect of age or environmental factors [9]. RAPD methods have been applied for clarification of the different medicinal species [10]. However, microscopic evaluation of the leaf constants and RAPD marker among four *J. multiflorum* cultivars existing in Thailand has never been evaluated. Therefore, the objectives of this study were to identify 4 *J. multiflorum* cultivars using leaf constants and RAPD marker to obtain the DNA fingerprinting and the genetic relationship of four *J. multiflorum* cultivars existing in Thailand.

MATERIALS AND METHODS

Plants materials

Both young and fresh mature leaves of 4 *J. multiforum* cultivars were collected from different locations in Thailand (each cultivar was collected from 3 locations, n=12). *Jasminum sambac* (L.) Aiton cultivar Mali La and *Pandorea jasminoides* (Lindl.) were used as outgroup plant in this study. All plant samples were authenticated by expert Assoc. Prof. Dr. Nijsiri Ruangrungsi and compared with herbarium specimens at Forest Herbarium Thailand (BKF). The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Microscopic evaluation of leaf constants

The microscopic appearances of leaves from 4 *J. multiforum* cultivars were examined under microscope. The central lamina of cleaned fresh mature leaves were cut into small pieces, soaked in Haiter bleach solution (Haiter bleach solution: distilled water, 1: 1) until chlorophyll was removed and then cleared by gently warming with chloral hydrate solution (4 g/ ml in distilled water) until the fragment was transparent. The transparent leaf sample was investigated under the photomicroscope (Zeiss Axioskop, Germany) with attached digital camera (Cannon Power shot A640) by wet mounting in glycerin. Microscopic evaluation was performed according to WHO guideline [11]. Stomatal density was determined by counting the number of stoma per square millimeter (mm^2) in 30 fields for individual sample. Stomatal index was obtained by the percentage of stomata from the total number of epidermal cells. The palisade ratio was obtained by counting the total number of palisade cells beneath four upper epidermal cells and divided by 4 [12]. The upper epidermal cell number, epidermal cell area, vein termination, vein islet and trichome number was also determined in 30 fields of each cultivar from 3 different locations. Leaf constants were calculated and expressed as mean ± standard deviation (SD) for each parameter.

Table 1 The average value (mean ± SD) of stomatal number, stomatal index, epidermal cell number, epidermal cell area, palisade ratio, vein termination number, vein islet number and trichome number of four *J. multiflorum* cultivars (n=12)

***J. multiflorum* cultivar**	**Stomatal number per mm^2**	**Stomatal index**	**Epidermal cell number per mm^2**	**Epidermal cell area (μm^2)**	**Palisade ratio**	**Vein termination number per mm^2**	**Vein islet number per mm^2**	**Trichome number per mm^2**	
								Adaxial surface	**Abaxial surface**
J. multiflorum (Maluli)	382.57 ± 22.53	11.07 ± 0.79	1733.24 ± 34.19	577.30 ± 8.17	1.69 ± 0.22	4.76 ± 1.24	4.36 ± 1.06	NF	5.71 ± 1.51
J. multiflorum (Maluli Sichomphu)	459.15± 51.74	11.94 ± 1.32	2837.91 ± 61.67	352.33 ± 15.46	1.62 ± 0.21	11.90 ± 1.87	6.70 ± 0.94	RF	6.55 ± 2.15
J. multiflorum (Maluli Phum)	404.66± 36.83	10.18 ± 0.65	3321.69 ± 66.43	301.07 ± 16.47	1.92 ± 0.22	7.55 ± 2.01	3.42 ± 1.12	12.31 ± 2.93	33.05 ± 2.73
J. multiflorum (Mali Ngachang)	350.53± 21.70	11.37 ± 0.75	2236.44 ± 51.47	447.09 ± 13.04	2.01 ± 0.14	14.11 ± 2.26	6.82± 1.41	NF	17.49 ± 3.66

RF = Rarely found (1-2 per mm^2), NF= Not found

Table 2 The sequence of the 8 RAPD primers and the number of PCR bands obtained from the four *J. multiflorum* cultivars

Primer	**Nucleotide sequence (5´ to 3´)**	**No. of bands**	**Size of bands**	**No. of polymorphic bands**	**Polymorphism (%)**
OPA-20	GTTGCGATCC	6	367-1563	4	66.66
OPB-04	GGACTGGAGT	7	336-1633	4	57.14
OPC-02	GTGAGGCGTC	11	213-1515	7	63.64
OPC-13	AAGCCTCGTC	4	513-1254	2	50.00
OPC-15	GACGGATCAG	10	419-1201	6	60.00
RAPD-09	CCTGGGCTTT	6	199-2328	3	50.00
A-29	GGTTCGGGAATG	8	377-1432	7	87.50
F-29	GCCGCTAATATG	7	398-1516	4	57.14
	Total	**59**	**199-2328**	**37**	**62.71**

RAPD analysis

DNA extraction and RAPD amplification

Fresh young leaves of 4 *J. multiflorum* cultivars and outgroup plants were grounded in liquid nitrogen and the total genomic DNA was individually extracted using a modified CTAB protocol [13]. Two microliter of extracted DNA were added to 20 µl of PCR reaction mixture containing of 1X amplification buffer, 4.0 mM $MgCl_2$, 0.2 mM of dNTPs, 1U of *Taq* DNA polymerase (Thermo Scientific, EU) and 0.4 µM RAPD primers (Operon Technologies, USA). The PCR amplification was performed using a DNA thermal cycler (AB Applied Biosystems, USA) with an initial denaturation of 95°C for 5 min, followed by 45 cycles of 95°C for 1 min, 36°C for 1 min, 72°C for 1 min and then followed by a final elongation of 72°C for 3 min. The PCR amplification products were separated on 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV transilluminator. RAPD fragments were photographed by gel documentation system (InGenius 3, USA).

RAPD data analysis

The RAPD bands were scored as 0 or 1 for the absence or presence of bands, respectively. Only clear and reproducible bands were scored. The molecular weights of the bands were estimated using 1 kb DNA markers (Thermo Scientific, EU). The similarity index was calculated from the data generated from Nei and Li's similarity index coefficient [14]. The dendrogram was constructed based on the similarity matrix data using the unweighted pair group method with arithmetic averages (UPGMA) clustering [15].

RESULTS AND DISCUSSION

Microscopic evaluation of leaf constants

Four *J. multiflorum* cultivars collected from different locations were investigated for leaf constants under microscope. According to the leaf constants, this is the first data of the leaf constant parameters from 4 *J. multiflorum* cultivars which has never been investigated. The leaf constant parameters were shown in Table 1. The type of stoma among 4 *J. multiflorum* cultivars is classified as anisocytic stomata in which the stoma surrounded by three subsidiary cells of which one is distinctly smaller than the other two [12] .

Leaf constants especially stomatal index is useful parameter in order to distinguish plant as well as palisade ratio is also useful for identification plant in species level [16]. This study confirmed the similarity in these parameters among selected *J. multiflorum* cultivars. However, trichome number was demonstrated to be useful to identify these 4 cultivars. Unicellular and multicellular trichome were found in 4 *J. multiflorum* cultivars. According to the present of trichome on adaxial surface of leaf, *J. multiflorum* caltivar Maluli Phum can be distinguished. Among the rest 3 cultivars, the number of trichome on abaxial surface (17.49 ± 3.66) describes *J. multiflorum* cultivar Mali Ngachang. *J. multiflorum* cultivar Maluli and Maluli Sichomphu can be characterized by vein termination number. Leaf constants evaluation is proved to be one of the methods capable in plant authentication even among cultivars. In the conventional authentication, herbal plants authentication depended on the experience of the collector's knowledge with the plants organoleptic characteristics. Therefore, development of reliable, easy and convenient methods is important. Microscopic method offers several advantages over conventional authentication including the effectiveness, simplicity and low cost. This method has been widely adopted as an official method in many international herbal pharmacopoeias [11].

RAPD analysis

Young fresh leaves of 4 *J. multiflorum* cultivars were used for DNA extraction due to low fibrous and low polyphenols and polysaccharides. The CTAB method for DNA extraction was found to be optimal to release the DNA from plant cell and suitable for PCR amplification.

Forty RAPD primers were screened and 8 primers (OPA-20, OPB-04, OPC-02, OPC-13, OPC-15, RAPD-09, A-29 and F-29) produced clear and reproducible bands (Table 2 and Figure 2). Due to each cultivar that was collected from 3 different localities showed the same pattern of RAPD profiles, an individual representative sample of each cultivar was selected. A total of 59 PCR fragments were amplified from 8 primers and the number of bands ranging from 4 to 11 with an average of 7.4 bands by each primer. The amplified products ranging from 199-2328 base pair in size. The lowest number of RAPD bands (4 bands) was detected in OPC-13 primer (Figure 2D), whereas the highest number of bands (11 bands) was generated from OPC-02 primer (Figure 2C). A Total of 59 amplified bands, 37 product bands were found to be polymorphic (62.71%). Primer A-29 (Figure 2G)

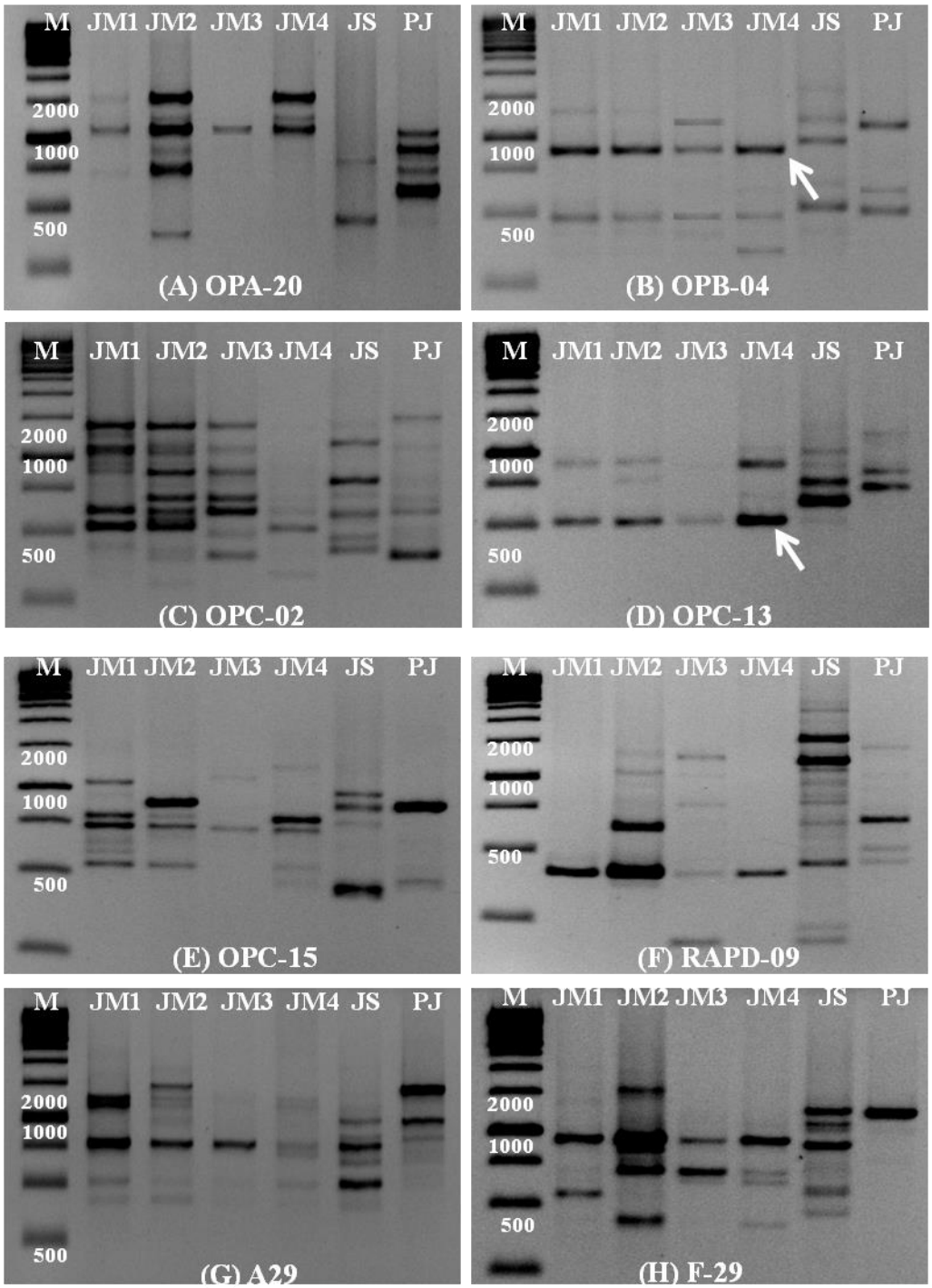

Figure 2 RAPD fingerprint of 4 *J. multiflorum* cultivars (JM1= *J. multiflorum* cultivar Maluli, JM2= *J. multiflorum* cultivar Maluli Sichomphu, JM3= *J. multiflorum* cultivar Maluli Phum and JM4= *J. multiflorum* cultivar Mali Ngachang and 2 outgroups (JS=*J. sambac* and PJ= *P. jasminoides*) obtained from primer OPA-20 (A), OPB-04 (B), OPC-02 (C), OPC-13 (D), OPC-15 (E), RAPD-09 (F), A-29 (G) and F-29 (H) primers, M= 1 kb marker, Arrow indicated monomorphic band

produced the highest percentage of polymorphism (87.50%) while OPC-13 (Figure 2D) and RAPD-09 (Figure 2F) produced the lowest percentage of polymorphism (50%). 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 (Figure 2B and 2D).

The pair-wise comparisons of the RAPD profiles based on both of the shared and unique

Table 3 Similarity matrix of the *J. multiflorum* cultivar and outgroup plants (*J. sambac* and *P. jasminoides*) generated using Dice similarity coefficient

***J. multiflorum* cultivar**	**Maluli**	**Maluli Sichomphu**	**Maluli Phum**	**Mali Ngachang**	***J. sambac***	***P. jasminoides***
Maluli	1.0000					
Maluli Sichomphu	0.7922	1.0000				
Maluli Phum	0.2587	0.4242	1.0000			
Mali Ngachang	0.2857	0.2583	0.4222	1.0000		
J. sambac	0.1183	0.0833	0.3131	0.1818	1.0000	
P. jasminoides	0.0774	0.1162	0.1428	0.1184	0.1250	1.0000

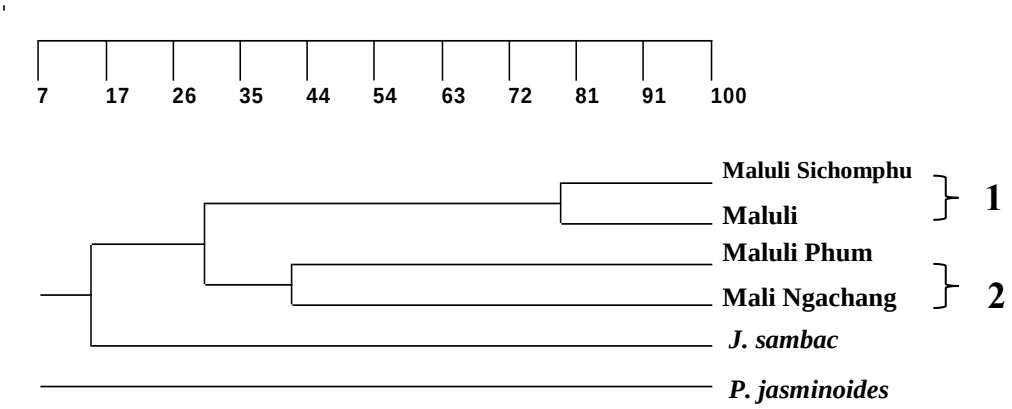

Figure 3 Dendrogram produced by UPGMA cluster analysis of RAPD data showing the genetic relationship among 4 *J. multiflorum* cultivars. The similarity scale is indicated at the top bar

amplification bands were used to generate a similarity index. Among 4 *J. multiflorum* cultivars including outgroup plants (*J. sambac* and *P. jasminoides*), Dice similarity index ranged from 0.2583–0.7922 (Table 3) indicated the low to moderate polymorphism. The highest similarity index (0.7922) was found between *J. multiflorum* cultivar Maluli Sichomphu and Maluli, whereas the lowest similarity index (0.2583) was found among *J. multiflorum* cultivar Mali Ngachang and Maluli Sichomphu.

A dendrogram was constructed according to the UPGMA cluster analysis using Dice similarity coefficient. Based on the UPGMA dendrogram could be divided into two clusters (Figure 3). Cluster I includes 2 cultivars of *J. multiflorum* cultivar Maluli Sichomphu and Maluli showing 0.7922 similarity index and cluster II includes 2 cultivars of *J. multiflorum* cultivar Maluli Phum and Mali Ngachang showing 0.4222 similarity index. Outgroup plant, *J. sambac* and *P. jasminoides* were clearly separated from *J. multiflorum*.

The molecular marker seems to be very effective tool for studying the DNA fingerprinting and genetic relationships between individual [17]. The limitation of RAPD is the reproducibility. Thus 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. According to the RAPD marker, this is also the first data of the molecular marker from 4 *J. multiflorum* cultivars that has never been investigated. Eight out of forty primers produced clear and reproducible bands. RAPD analysis has been widely used to differentiate between medicinal species and their close relatives, including *Phyllanthus* spp. [18], *Jasminum* spp. [19] and *Cassia* spp. [20, 21]. Indeed, RAPD analysis has been successfully used for taxonomic classification and study of genetic diversity in plants [22].

CONCLUSIONS

Based on the leaf constants and RAPD analysis of 4 *J. multiflorum* cultivars, the data obtained from this study provides the useful information and practical methods for medicinal plant identification. This is the first data of the leaf constant parameters

and RAPD marker from 4 *J. multiflorum* cultivars. RAPD technique is a useful tool for studying genetic diversity and genetic relationship between and within the *Jasminum* species. For further research, SCAR marker should be developed for identification of plant in *J. multiflorum* cultivars.

ACKNOWLEDGMENTS

This study was supported from the 90th Anniversary of Chulalongkorn University fund (Ratchadaphisaksomphot Endowment Fund). The authors would like to express sincerely grateful to College of Public Health Sciences, Chulalongkorn University for instrument supports and valuable information.

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