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Phylogenetic Relationships of Selected *Kaempferia* Plants in Thailand Based on RAPD Marker

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ABSTRACT

Random amplified polymorphic DNA (RAPD) marker was applied for detecting the phylogenetic relationships among five selected *Kaempferia* plants existing in Thailand. A total of 40 random primers were preliminary screened, six primers produced clear and reproducible polymorphic bands. Eleven to nineteen products were amplified, with an average of 15.5 bands by each primer. A total of 93 scorable bands ranging from 159 to 2464 base pair in size were amplified, among which 47 products were found to be polymorphic. The similarity index (SI) ranged from 0.1865-0.6928. The dendrograms were constructed using unweighted pair group method with arithmetic averages (UPGMA). The results from the cluster diagram could be divided into two main groups and the phylogenetic relationships were associated with the morphological characterization.

Keywords: *Kaempferia galanga*, *Kaempferia larsenii*, *Kaempferia marginata*, *Kaempferia rotunda*, *Kaempferia parviflora*, Zingiberaceae

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INTRODUCTION

Rhizomatous herbs in the genus *Kaempferia*, belong to the family Zingiberaceae, are those of important medicinal plants in Thailand. They are small herbaceous plants with short rhizome and tuberous root. They have few radical leaves. The upper surface of a leaf is often marbled and the lower side is dark green to purple. *Kaempferia* plants are ethnomedically used in the treatment of pneumonia, bronchial complaints, abdominal illness, dysentery, diarrhoea, leukorrhea, wound and insect bite [1-3]. Because of the synonym in vernacular name of plants in the genus *Kaempferia*, makes it difficult for the identification of plants in this genus. Moreover, the genus *Kaempferia* is morphologically closely related to the genera *Boesenbergia, Scaphochlamys* and *Caulokaempferia* [4-5]. In addition, the taxonomic identification to the species level without the floral parts is also difficult [5].

DNA technologies are reliable and powerful tools for identification of taxa at various taxonomic levels (e.g., species, subspecies, variety, strain) as they provide consistent results irrespective of age, tissue origin, physiological conditions, environmental factors, harvest, storage, and processing of samples [6]. The random amplified polymorphic DNA (RAPD) technique [7] is a popular tool in genetic studies. RAPD markers provide a rapid, inexpensive and effective system for studying plant genetic relationships [8]. RAPD technique has been used to distinguish several plants in Zingiberaceae such as *Boesenbergia* [9], *Kaempferia* [9-10] and *Curcuma* [8, 11-12].

Thus, the aim of this study was to evaluate the phylogenetic relationships of five selected *Kaempferia* plants existing in Thailand using RAPD fingerprints.

MATERIALS AND METHODS

Plant materials

Fresh rhizomes of five selected *Kaempferia* (*Kaempferia galanga*, *K. marginata*, *K. larsenii, K. parviflora,* and *K. rotunda*) were collected in June 2009 from different locations in Thailand **(Table 1)**. *Zingiber montanum* and *Citrus hystrix* were used as outgroup plant in this study. All sample plants were grown at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand, for 1-2 months. Young leaves of each plant were used for DNA isolation.

Table 1. List of the plant samples used in this study

Plant name	Code	Locality
Kaempferia galanga	KG	Chiang Mai
K. larsenii	KL	Ubon Ratchathani
K. marginata	KM	Prachine Buri
K. parviflora	KP	Ratchaburi
K. rotunda	KR	Ratchaburi
Zingiber montanum	ZM	Ratchaburi
Citrus hystrix	CH	Pathum Thani

DNA isolation and random amplified polymorphic DNA (RAPD) fingerprinting

Fresh leaf of each plant was ground in liquid nitrogen with mortar and pestle to obtain a fine powder. Genomic DNA was isolated from the fine powder using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

The RAPD reaction was carried out in 20 µl containing 1-2 µl of genomic DNA, 1X amplification buffer, 5 mM $MgCl_2$, 0.4 mM of each dNTP, 1.25 U of *Taq* DNA polymerase (Fermentas, Canada) and 0.4 µM random primers (Eurofins MWG Operon, Germany). The amplification was performed using a DNA thermal cycler (Applied Biosystems, USA) with an initial pre-denaturation at 95°C for 2 min, denaturation at 95°C for 45 sec, annealing at 37°C for 1 min, extension at 72°C for 2 min with 45 cycles and final extension at 72°C for 5 min. The RAPD products were separated on 1.5% agarose gel in TBE buffer and stained with ethidium bromide. The RAPD fragments were photographed using a UV transilluminator and analyzed with a gel documentation system (Syngene, 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 as 1. The standard DNA marker (1 kb GeneRuler, Fermentas, Canada) was used to assign the size of each RAPD fragment. The similarity index was calculated from the data that was generated using Dice similarity index coefficient [13]. The dendrogram was constructed based on the similarity matrix data using the unweighted pair group method with arithmetic averages (UPGMA), clustering by GeneTool and GeneDirectory software.

RESULTS

Forty random primers were initial screened, only six primers (OPA 15, OPY 15, OPY 20, RAPD 05, RAPD 18 and RAPD-F 29) produced clear and reproducible polymorphic bands in all plant samples. Eleven to nineteen PCR products were amplified, with an average of 15.5 bands by each primer. The highest number of RAPD bands (19 bands) was generated from OPY-20 while the lowest (11 bands) was generated from OPY 15. A total of 93 amplified bands ranging from 159 to 2464 base pair in size were amplified, among which 47 product bands were found to be polymorphic. Primer RAPD-F 29 produced the highest percentage of polymorphism (86.67%) while OPA 15 produced the lowest percentage of polymorphism (27.78%) **(Table 2)**.

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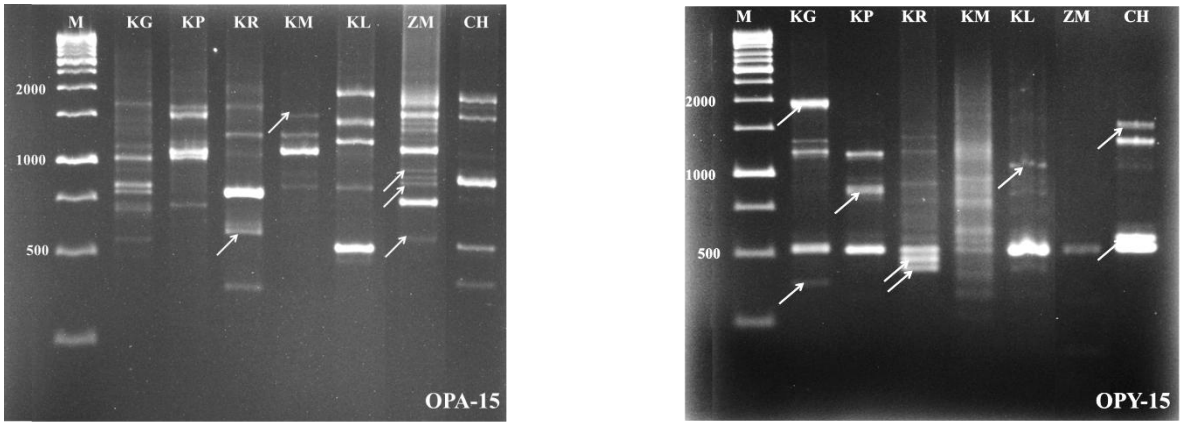
Table 2. The sequence of the oligonucleotide primers used for the RAPD analysis and the number of PCR products obtained from five *Kaempferia* plants and outgroup plants

Primer	Nucleotide sequence (5´ to 3´)	No. of bands	Size of bands	No. of polymorphic bands	Polymorphism (%)
OPA 15	TTCCGAACCC	18	380 - 1875	5	27.78
OPY 15	AGTCGCCCTT	11	366 – 1888	8	72.73
OPY 20	AGCCGTGGAA	19	159 - 2416	7	36.84
RAPD 05	TTCCGGGTGC	13	458 – 2115	8	61.54
RAPD 18	CCACGGTAGC	17	248 – 2409	6	35.29
RAPD-F 29	GCCGCTAATATG	15	595 – 2464	13	86.67

According to the six primers that produced clear and reproducible polymorphic bands, the OPA 15 primer produced the polymorphic bands of 584 bp in *K. rotunda*, 1399 bp in *K. marginata* and 553, 877 and 929 bp in *Z. montanum* (Figure 1A). The OPY-15 primer produced the polymorphic bands of 366 bp and 1888 bp in *K. galanga*, 846 bp in *K. parviflora*, 419 and 456 bp in *K. rotunda*, 1031 bp in *K. larsenii*, 519 and 1402 bp in *C. hystrix* (Figure 1B). The OPY-20 primer produced the polymorphic bands of 471 bp in *K. galanga*, 1191 bp in *K. marginata*, 932, 2035 and 2416 bp in *K. larsenii*, 159 bp in *Z. montanum* and 298 bp in *C. hystrix* (Figure 1C). The RAPD-05 primer produced the polymorphic bands of 458 and 702 in *K. galanga*, 1078 bp in *K. rotunda*, 473 bp in*K. marginata*, 595, 656 and 1399 bp in *Z. montanum* and 554 bp in *C. hystrix* (Figure 1D). The RAPD 18 primer produced the polymorphic bands of 340 bp in *K. galanga*, 248 and 383 bp in *K. rotunda*, 407 and 2409 bp in *K. larsenii* and 1606 bp in *Z. montanum* (Figure 1E). The RAPD-F 29 primer produced the polymorphic bands of 1419 bp in *K. galanga*, 595 bp in *K. parviflora*, 1167 and 1644 bp in *K. rotunda,* 1125 and 1858 bp in *K. marginata*, 800 bp in *K. larsenii*, 691 bp in *Z. montanum*, 653, 742, 1377, 1759 and 2464 bp in *C. hystrix* (Figure 1F). The RAPD-F 29 primer produced polymorphic bands in all plant samples. Moreover, this primer generated the approximately 1000 bp characteristic band of zingiberaceous plants but not observed in *C. hystrix* (Figure 1F).

(A) **(B)**



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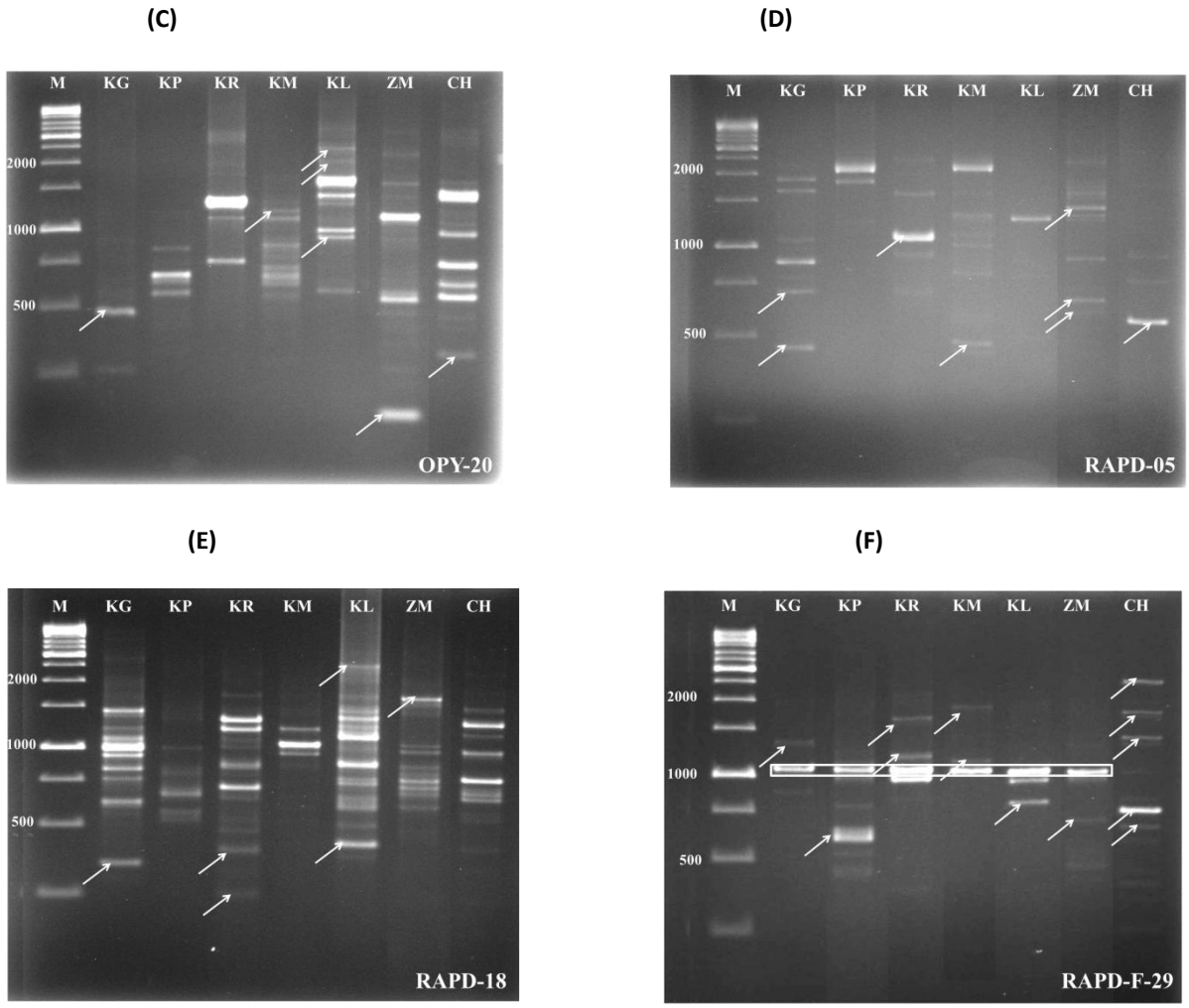

Figure 1. RAPD fingerprint of five *Kaempferia* plants and outgroup plants obtained from the OPA-15 (A), OPY-15 (B), OPY-20 (C), RAPD-05 (D), RAPD-18 (E) and RAPD-F-29 (F) primers. Abbreviations of the plant samples are according to according to the codes used in Table 1. M: GeneRuler 1 kb (size shown in bp). The polymorphic bands of each plant sample are indicated with arrows.

The pair-wise comparisons of the RAPD profiles based on both of the shared and unique amplification bands were used to generate a similarity index. Among five *Kaempferia* species including outgroup plants, Dice similarity index ranged from 0.1865 to 0.6928 (Table 3). The highest genetic similarity index (0.6928) was found between *K. marginata* and *K. galanga*, whereas the lowest genetic similarity index (0.1865) was found among *K. galanga* and *C. hystrix*.

A dendrogram was constructed according to the UPGMA cluster analysis using Dice similarity coefficient. The UPGMA dendrogram could be divided into two clusters (Figure 2). Cluster I includes 2 species of *K. marginata* and *K. galangal* showing the similarity index 0.6928, and cluster II includes 3 species of *K. parviflora, K. rotunda* and *K. larsenii* showing 0.3465 to 0.6600 similarity index. Outgroup plants, *Z. montanum* and *C. hystrix*, were clearly separated from *Kaempferia* plants.

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Table 3. Similarity matrix of *Kaempferia* plants and outgroup plants generated using Dice similarity coefficient

Species	*K. marginata*	*K. galanga*	*K. larsenii*	*K. rotunda*	*K. parviflora*	*Z. montanum*	*C. hystrix*
K. marginata	1.0000						
K. galanga	0.6928	1.0000					
K. larsenii	0.3307	0.2865	1.0000				
K. rotunda	0.4588	0.4027	0.5939	1.0000			
K. parviflora	0.3511	0.3094	0.6337	0.6600	1.0000		
Z. montanum	0.3200	0.2508	0.3652	0.3502	0.3465	1.0000	
C. hystrix	0.2381	0.1865	0.2231	0.2777	0.2840	0.2347	1.0000

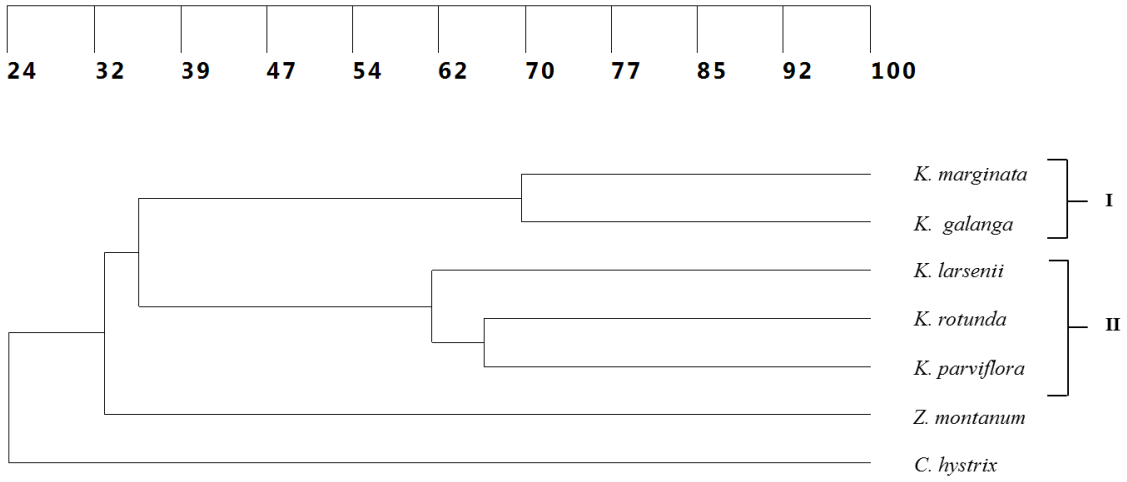

Figure 2. Dendrogram produced by UPGMA cluster analysis of RAPD data showing the genetic relationship among five *Kaempferia* plants and outgroup plants

DISCUSSION AND CONCLUSION

RAPD marker provides a rapid, inexpensive and effective system for studying genetic relationships in various organisms due to their advantage no need of prior knowledge of DNA sequence, small amount of DNA is require and can also assay for many loci simultaneously. [8]. However, the limitation of RAPD is the reproducibility and cannot differentiate dominant homozygote from heterozygote. To concern about reproducibility, optimization of RAPD component is necessary. RAPD marker has been used to analyze the genetic diversity in several plant species such as *Cuurcuma* spp. [14], *Ocimum* spp. [15], *Asimina triloba* [16], *Olea europaea* [17] and *Punica granatum* [18]. Moreover, RAPD marker has been shown the correlation with morphological characteristics in *Boesenbergia* spp. [9], *Derris* spp. [19], *Lavatera* spp. [20], *Pisum* spp. [21], *Quercus* spp. [22] and *Thunbergia* spp. [23].

In this present study, we investigated the phylogenetic relationship of 5 *Kaempferia* plant species using RAPD marker. Six out of forty primers generated the unique RAPD profiles. According to the UPGMA dendrogram, 5 *Kaempferia* species could be divided into

two clusters. Cluster I includes 2 species of *K. marginata* and *K. galangal* and cluster II includes 3 species of *K. parviflora, K. rotunda* and *K. larsenii*. Previous study support our results that *K. marginata* and *K. galanga* were clustered into the same group based on the chloroplast DNA sequences [5]. Furthermore, the results were correlated with the morphological characteristic. *K. marginata* and *K. galanga* which clustering into the first group has ovate-suborbicular, unequal-side, upper surface glabrous, undersurface hairy, sessile leaves with inflorescence totally enclosed in the two leaf-sheaths and white flowers [4, 24]. Moreover, Picheansoonthon and Koonterm noted that *K. marginata* may be conspecific to *K. galanga* [4]. *K. parviflora, K. rotunda* and *K. larsenii* which clustering into second group have ellictic or oblong leaves, linear leaf blade and pedunculated inflorescence with white or light purple flowers [4, 24]. The leaves surface and the length of petiole and peduncle can be applied for differentiation *K. parviflora, K. rotunda* and *K. larsenii*. *K. rotunda* and *K. parviflora* have hairy in undersurface leaves, longer petiole and peduncle while *K. larsenii* has glabrous leaves, shorter petiole and sessile inflorescence [4, 24]. The polymorphic banding pattern which is the unique band derived from RAPD marker that found in all *Kaempferia* species can be developed as RAPD-derived sequence characterized amplified regions (SCAR) marker development for rapid detection of *Kaempferia* species.

In conclusion, out investigation affirmed the ability of RAPD fingerprint to differentiate among 5 *Kaempferia* plants and also revealed the genetic relationships of these plants. Furthermore, sequence characterized amplified regions (SCARs) could be further developed to differentiated plants that have similar morphology.

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Author Disclosure Statement

The authors declear that there are no conflicts of interest.

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