Original Article

Phylogenetic relationships of *Kaempferia* plants based on inter-simple sequence repeat fingerprints

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Abstract

Plants in genus *Kaempferia* (Zingiberaceae) have been used as folk medicine in several countries. Identification and classification of *Kaempferia* plants are difficult because of the morphological similarity between species. In this study, phylogenetic relationships of *Kaempferia* species were employed by inter-simple sequence repeat (ISSR) fingerprints. Ten ISSR primers of 45 ISSR primers produced 186 bands with an average 19 bands per primer. The similarity index (SI) ranged from 0.0023-0.9950. A dendrogram was constructed using unweighted pair-group method of the arithmetic average (UPGMA). The results showed that the *Kaempferia* species could be divided into two main groups. The phylogenetic relationships were associated with the morphological characteristics.

Keywords: *Kaempferia*, Zingiberaceae, inter-simple sequence repeat fingerprints

1. Introduction

The genus *Kaempferia* is a medium-sized rhizomatous herb belonging to the Zingiberaceae family. *Kaempferia* is comprised of approximately 40 species distributed in monsoonal tropical Asia (Skornickova & Newman, 2015), of which 29 are found in Thailand (Pooma & Suddee, 2014). In Thailand, *Kaempferia* plants are ethnomedically used in the treatment of flatulence, fever, gastric ulcer, leucorrhoea, oedema, and healing of wounds (Chuakul, 2005; Chuakul & Boonpleng, 2003, 2004; Maneenoon *et al.*, 2015; Nuammee *et al.*, 2012; Suksri *et al.*, 2005; Tangjitman *et al.*, 2015).

However, the taxonomic identification and classification of these plants based on morphological characteristics is not always satisfactory because of their similar appearance within the genus and also with other closely related genera, i.e. *Boesenbergia*, *Scaphochlamys*, *Caulokaempferia*, and *Curcuma* (Kitamura *et al.*, 2007; Picheansoonthon & Koonterm, 2008; Syamkumar & Sasikumar, 2007; Techaprasan *et al.*, 2010; Vanijajiva *et al.*, 2005; Zou *et al.*, 2011).

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Nowadays, molecular markers are valuable tools in the characterization and evaluation of genetic diversity within and between species and populations (Heubl, 2010; Semagn *et al.*, 2006; Tharachand *et al.*, 2012). The inter-simple sequence repeat (ISSR) technique is a fast and inexpensive technique for the characterization of genetic relatedness among populations (Dogan *et al.*, 2016; Prevost & Wilkinson, 1999). It is the marker of choice for the amplification of variations of genomic sequences between microsatellites using a single primer of nucleotide repeat units. ISSR employs simple sequence repeats in the primers so that it does not require sequence information for primer design, which is a major advantage of ISSR analysis (Son *et al.*, 2012). The ISSR marker has been used in phylogenetic analysis in several plants such as *Alium* (Hao *et al.*, 2002; Son *et al.*, 2012), *Oryza* (Joshi *et al.*, 2000); *Phyllanthus* (Rout & Aparajita, 2010),*Matthiola* (Dagan *et al.*, 2016), and *Klasea* (Dogan *et al.*, 2015). However, the number of studies of the genetic relationships of *Kaempferia* species using the ISSR marker is quite limited.

Thus, the aims of this study were to investigate the genetic diversity and phylogenetic relationships of *Kaempferia* species on the basis of ISSR fingerprinting analysis.

2. Materials and Methods

2.1 Plant materials

Fresh rhizomes of 11 accessions of *Kaempferia* belonging to 10 species were collected between May and July 2015 from different locations (9 accessions from Thailand and 2 accessions from Laos). Four Zingiberaceous plants; *Curcuma aromatica*, *Boesenbergia rotunda*, *Zingiber montanum* (tribe Zingibereae), *Alpinia galanga* (tribe Alpinieae), and *Citrus hystrix* (Rutaceae) were used as outgroup plants in this study. The details of plant samples used in the study are shown in Table 1. The plant samples

Table 1.   Details of plant samples used in this study.

Plant samples	Locality	Code
Kaempferia angustifolia Roscoe	Prachin Buri	KA
K. elegans (Wall.) Baker	Prachin Buri	KE
K. galanga L.	Chiang Mai	KG
K. laotica Gagnep.	Laos	KL
K. larsenii Sirirugsa	Ubon Ratchathani	KN
K. marginata Carey ex Roscoe	Prachin Buri	KM
K. parviflora Wall. ex Baker (cultivar type)	Prachin Buri	KP
K. parviflora Wall. ex Baker (wild type)	Laos	KP2
K. pulchra Ridl.	Kanchanaburi	KC
K. rotunda L.	Ratchaburi	KR
K. siamensis Sirirugsa	Sakon Nakhon	KS
Curcuma aromatica Salisb.	Chiang Mai	CA
Zingiber montanum (J.Koenig) Link ex A.Dietr.	Bangkok	ZM
Alpinia galanga (L.) Willd.	Bangkok	AG
Boesenbergia rotunda (L.) Mansf.	Bangkok	BR
Citrus hystrix DC.	Pathum Thani	CH

were authenticated by Assist. Prof. Dr. Thaya Jenjittikul (Department of Plant Science, Faculty of Science, Mahidol University, Thailand). The voucher specimens of these plants have been deposited at Department of Pharmacognosy, Faculty of Pharmacy, Rangsit University, Thailand. All plant samples were employed for cultivation at the Department of Pharmacognosy, Faculty of Pharmacy, Rangsit University, Bangkok, Thailand, for 4-6 weeks. Morphological characters of the cultivated plants were recorded, i.e. leaf position, leaf shape, leaf base, leaf apex, and position of inflorescence.

2.2 DNA isolation

Fresh young leaf of each plant was ground in liquid nitrogen with mortar and pestle to obtain a fine powder. Total genomic DNA was isolated from the fine powder using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA quantifications were checked by 1.5% agarose gel electrophoresis. Genomic DNA was kept at -20 °C for ISSR analysis.

2.3 Inter-simple sequence repeat (ISSR) amplification

The ISSR reaction was carried out in 25 µl containing 2 µl of genomic DNA, 1X amplification buffer, 5 mM $MgCl_2$, 0.4 mM of each dNTP, 1 U of *Taq* DNA polymerase (Fermentas, Canada), and 0.4 µM ISSR 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 1 min, denaturation at 95 °C for 45 sec, annealing at 45 °C for 45 sec, extension at 72 °C for 1 min with 35 cycles and final extension at 72 °C for 5 min. The ISSR products were separated alongside a molecular weight marker (1 kb Gene-Ruler, Fermentas, Canada) on 1.5% agarose gel in TBE buffer and stained with ethidium bromide. The ISSR fragments were photographed using a UV transilluminator and analyzed with a gel documentation system (Syngene, USA).The PCR amplifications were repeated at least three times in the same condition.

2.4 ISSR data analysis

The ISSR 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 similarity index was calculated from the data generated using Dice similarity index coefficient (Nei & Li, 1979). The dendrogram was constructed based on the similarity matrix data using the unweighted pair group method with arithmetic averages (UPGMA), clustering by FreeTree and TreeView software with 1000 bootstrap (Pavlicek *et al.*, 1999).

3. Results and Discussion

A total of 45 ISSR primers were used for initial screening;10 primers produced 186 clear and reproducible bands in all plant samples. The total number of amplified products ranged from 12-24 bands with an average of 19 bands by each primer. The highest number of ISSR bands (24 bands) was generated from ISSR-38 primer while the lowest number of bands (12 bands) was from ISSR-40. The

approximate range of band size was 223-2505 bp. The percentage of polymorphism ranged from 9.25% to 41.67% as demonstrated by ISSR-27 and ISSR-40, respectively (Table 2). Change in the amplified products using ISSR primer can arise through the insertions/deletions or the loss of primer binding sites which generated different banding patterns.

Table 2. The sequence of ISSR primers and the number of PCR products obtained from plant samples.

Primer	Nucleotide sequence (5´ to 3´)	No. of bands	Size of bands (bp)	Polymorphism (%)
ISSR 02	AGA GAG AGA GAG AGC	18	252-1456	16.67
ISSR 08	GTG TGT GTG TGT GTC	18	304-2505	33.33
ISSR 19	GAG AGA GAG AGA GAG GC	22	264-2472	13.64
ISSR 27	AGA GAG AGA GAG AGA CYT	21	259-1903	9.25
ISSR 28	AGA GAG AGA GAG AGA GYA	17	237-1586	17.65
ISSR 37	TGT GTG TGT GTG TGT GRC	16	267-1661	25.00
ISSR 38	AGC AGC AGC AGC AGC AGC	24	223-1322	25.00
ISSR 39	ATG ATG ATG ATG ATG ATG	22	242-2108	22.73
ISSR 40	CTC CTC CTC CTC CTC CTC	12	278-1676	41.67
ISSR 43	GGG TGG GGT GGG GGT	17	284-1727	23.53
	Total	186	223-2505	

According to 10 primers that produced clear and reproducible bands, the ISSR-27 primer generated the approximately 1000 bp characteristic band of all *Kaempferia* species and four Zingiberaceous outgroup plants, this band was not observed in *C. hystrix* (Figure 1). Therefore, this band may be used as the characteristic band for Zingiberaceous plants.
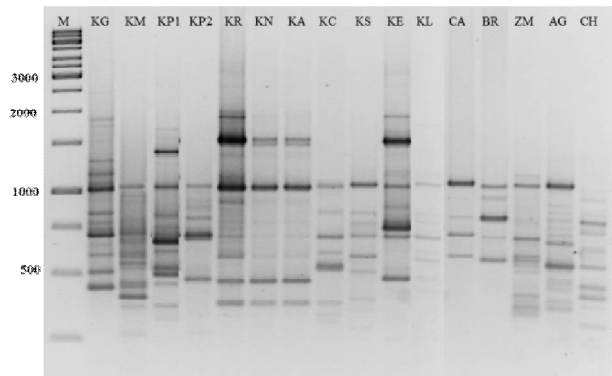

Figure 1. ISSR fingerprint of 11 *Kaempferia* and outgroup plants obtained from the ISSR-27 primer. Abbreviations of the plant samples are according to codes used in Table 1. M: GeneRuler 1 kb (size shown in bp).

Since ISSR are dominant markers, the amplified bands are scored as dialleic. The pair-wise comparisons of the ISSR profiles based on both the shared and unique amplification bands were used to generate a similarity index. Among 11 accessions of *Kaempferia* plants including 5 outgroup plants, Dice similarity index ranged from 0.0023 to 0.9950 (Table 3). The highest genetic similarity index (0.9950) was found between *K. parviflora* cultivar and wild type, whereas the lowest (0.0023) was found between *K. elegans* and *Citrus hystrix*.

A dendrogram was constructed according to the UPGMA cluster analysis using the Dice similarity coefficient. Based on the dendrogram, *Kaempferia* species were categorized into two major groups. Cluster I and II were divided into two subgroups Ia/ Ib and IIa/IIb, respectively. Subgroup Ia, consisted of *K. elegans* and *K. parviflora* (cultivar andwild type), with a similarity index range of 0.9684-0.9950. Subgroup Ib included *K. larsenii*, *K. rotunda*, and*K. angustifolia*, with a similarity index range of 0.9849-0.9920. *K. siamensis*, *K. galangal*, and *K. marginata* were clustered into subgroup IIa with a similarity index range of 0.9170-0.9793, whereas *K. pulchra* and *K. laotica* were

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

	KG	KM	KS	KC	KL	KP1	KP2	KE	KR	KA	KN	ZM	AG	CA	BR	CH
KG	1.0000															
KM	0.9793	1.0000														
KS	0.9170	0.9397	1.0000													
KC	0.8251	0.8053	0.8120	1.0000												
KL	0.8957	0.8755	0.8880	0.9106	1.0000											
KP1	0.4585	0.4688	0.4924	0.5892	0.5359	1.0000										
KP2	0.4622	0.4646	0.4886	0.5938	0.5399	0.9950	1.0000									
KE	0.4603	0.4706	0.4867	0.5914	0.5379	0.9756	0.9684	1.0000								
KR	0.6015	0.6022	0.6202	0.6619	0.6528	0.7653	0.7573	0.7613	1.0000							
KA	0.6087	0.6093	0.6272	0.6690	0.6597	0.7588	0.7508	0.7548	0.9920	1.0000						
KN	0.6081	0.6087	0.6127	0.6547	0.6456	0.7468	0.7386	0.7492	0.9849	0.9849	1.0000					
ZM	0.1504	0.1747	0.1772	0.1732	0.1765	0.2222	0.2162	0.2154	0.2324	0.2324	0.2278	1.0000				
AG	0.0566	0.0644	0.0717	0.0669	0.0716	0.0978	0.0955	0.0928	0.1050	0.1075	0.1029	0.1947	1.0000			
CA	0.2097	0.2197	0.2275	0.2115	0.2308	0.2531	0.2504	0.2500	0.2838	0.2838	0.2750	0.2066	0.6011	1.0000		
BR	0.2161	0.2222	0.2262	0.2251	0.2258	0.2547	0.2487	0.2517	0.2782	0.2782	0.2762	0.2202	0.6075	0.5035	1.0000	
CH	0.0024	0.0100	0.0032	0.0167	0.0098	0.0170	0.0076	0.0023	0.0514	0.0079	0.0947	0.0812	0.0694	0.0326	0.0212	1.0000

clustered into subgroup IIb with a 0.9106 similarity index. The separation of all subgroups and two major groups received 100% bootstrap support. Four Zingiberaceous outgroup plants, *Zingiber montanum*, *Curcuma aromatica*, *Boesenbergia rotunda*, and *Alpiniagalanga*, were completely separated from the *Kaempferia* species with 100% bootstrap support. In addition, *Citrus hystrix*, a dicotyledon plant, was completely separated from all Zingiberaceous plants with 100% bootstrap support (Figure 2). The results were similar to

those previously reported. Based on ISSR fingerprints, *K. angustifolia* and *K. rotunda* were clustered into the same group (Siriluck *et al.*, 2014) while based on RAPD finger-prints and chloroplast DNA sequences, *K. marginata* and *K. galanga* were clustered together (Techaprasan *et al.*, 2010; Theanphong *et al.*, 2013). In addition, *K. parviflora* and *K. elegans* were clustered into the same group on the basis of chloroplast DNA sequences (Techaprasan *et al.*, 2010).
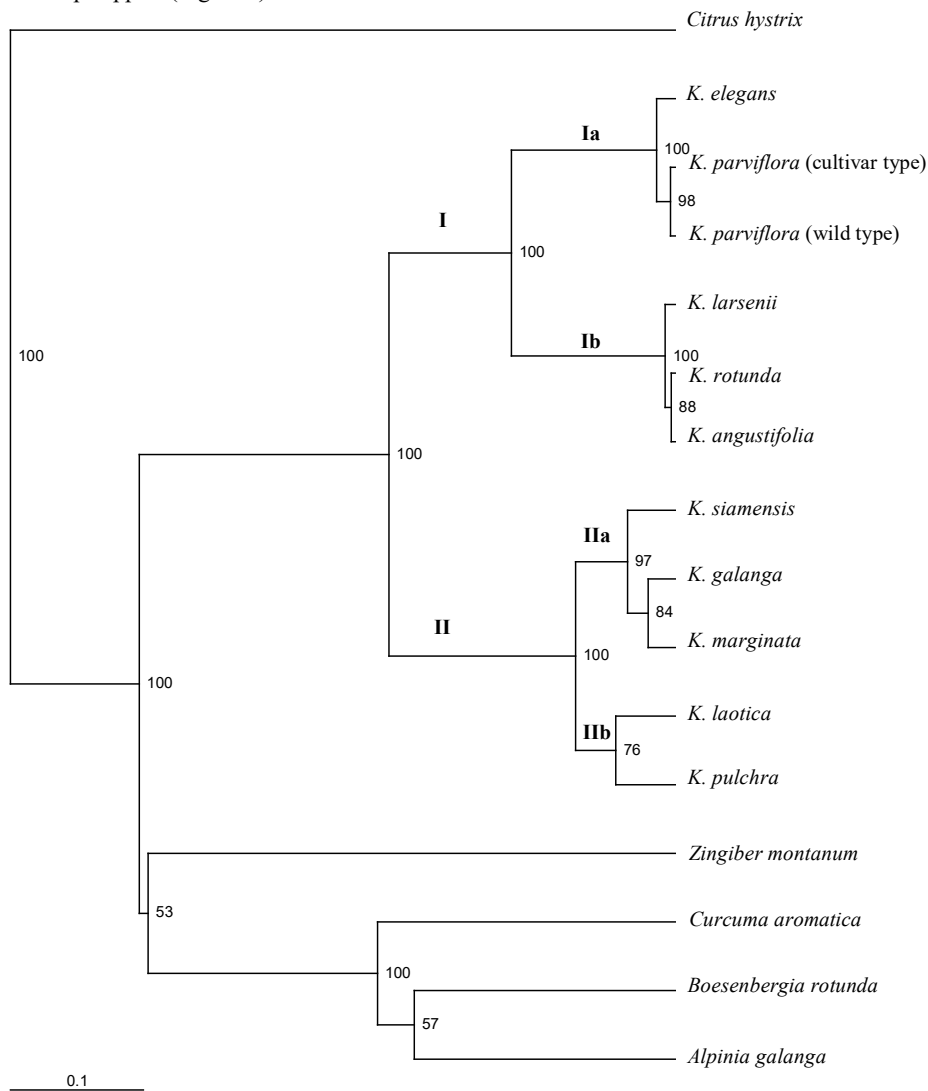

Figure 2.   Dendrogram produced by UPGMA cluster analysis of ISSR data showing the genetic relationship among 11 *Kaempferia* plants and outgroup plant.

The results obtained from this study were also correlated with the morphological characteristics. *Kaempferia* plants incluster I were 6-30 cm tall and they had erect elliptic oblong leaves and inflorescence rising between the two innermost leaf-sheaths or rising from the rhizome together with a 1-10 cm petiole length. *K. siamensis*, *K. galanga*, *K. marginata*, *K. pulchra*, and *K. laotica* which were clustered into the second group were 2-10 cm tall and had horizontal elliptic-suborbicular leaves and inflorescence totally enclosed

in the two leaf-sheaths. The important morphological characteristics of each cluster are shown in Table 4. The resultswere similar to those reported by Sirirugsa (1992). Based on the morphological characteristics described by Sirirugsa, 15 *Kaempferia* species from Thailand were divided into two main groups. Eight *Kampferia* species that included *K. spoliata*, *K. larsenii*, *K. flallax*, *K. filifolia*, *K. elegans*, *K. parviflora*, *K. rotunda*, and *K. angustifolia* were clustered in the first group. These plants have erect leaves together with

Table 4. The important morphological characters of the plants in group I and II.

Morphological characters	Group I		Group II	
	Subgroup Ia	Subgroup Ib	Subgroup IIa	Subgroup IIb
Height	6-20 cm	6-30 cm	2-5 cm	2-10 cm
Leaf position	Erect	Erect	Horizontal	Horizontal
Leaf shape	Elliptic	Elliptic	Elliptic or suborbicular	Elliptic or suborbicular
Leaf apex	Acute	Acute	Acute or mucronate	Acute or mucronate
Leaf base	Cuneate	Cuneate	Rounded-cuneate	Rounded-cuneate
Petiole length	1-10 cm	Sessile or 1-2 cm	Sessile	Sessile or 3-12 cm
Position of inflorescence	Rising between the two innermost leaves or enclose by the two innermost leaf-sheaths	Rising between the two innermost leaves or enclose by the two innermost leaf-sheaths	Enclose by the two innermost leaf-sheaths	Enclose by the two innermost leaf-sheaths
Anther crest apex	Entire	Deeply divided	Entire	Deeply divided

elliptic-liner, fillilorm, lanceolate or orbicular leaf blades. *K. siamensis*, *K. marginata*, *K. galanga*, *K. pulchra*, *K. glauca*, *K. roscoeana*, and *K. laotica* were clustered in the second group which has horizontal leaves.

The results indicated that the genetic relationships through ISSR fingerprints together with morphological characteristics provide a reliable method for identification of plants that have similar morphological characteristics.

4. Conclusions

The phylogenetic relationships of 11 accessions of *Kaempferia* plants belonging to 10 species from Thailand and Laos were determined by ISSR fingerprints. Forty-five ISSR primers were screened, of which 10 primers produced clear and reproducible bands. According to the ISSR profiles, the *Kaempferia* plants were divided into two major groups. ISSR profiles showed that the ISSR-27 primer generated characteristic bands of Zingiberaceous plants. Therefore, the ISSR-27 primer could be further developed to authenticate Zingiberaceous plants. The ISSR marker is a reliable and cost effective technique in addition to assessing genetic diversity in Zingiberaceous plants. Furthermore, sequence characterized amplified regions could be further developed to quality control of herbal medicines anddifferentiation of plants in the *Kaempferia* genus. Moreover, this is the first report on ISSR fingerprints of *Kaaempferia* plants. The data might be used as additional information for plant conservation and plant breeding for the *Kaempferia* genus.

Acknowledgements

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