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Genetic variation of *Kaempferia* (Zingiberaceae) in Thailand based on chloroplast DNA (*psbA-trnH* and *petA-psbJ*) sequences

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ABSTRACT. Genetic variation and species authentication of 71 *Kaempferia* accessions (representing 15 recognized, six new, and four unidentified species) found indigenously in Thailand were examined by determining chloroplast *psbA-trnH* and partial *petA-psbJ* spacer sequences. Ten closely related species (*Boesenbergia rotunda*, *Gagnepainia godefroyi*, *G. thoreliana*, *Globba substrigosa*, *Smithatris myanmarensis*, *S. supraneanae*, *Scaphochlamys biloba*, *S. minutiflora*, *S. rubescens*, and *Stahlianthus* sp) were also included. After sequence alignments, 1010 and 865 bp in length were obtained for the respective chloroplast DNA sequences. Intraspecific sequence variation was not observed in *Kaempferia candida*, *K. angustifolia*, *K. laotica*, *K. galanga*, *K. pardi* sp nov., *K. bambusetorum* sp nov., *K. albomaculata* sp nov., *K. minuta* sp nov., *Kaempferia* sp nov. 1, and *G. thoreliana*, for which more than one specimen was available. In contrast, intraspecific sequence polymorphisms were observed in various populations of *K. fallax*, *K. filifolia*, *K. elegans*, *K. pulchra*, *K. rotunda*, *K. marginata*, *K. parviflora*, *K. larsenii*, *K. roscoeana*, *K. siamensis*, and *G. godefroyi*.

A strict consensus tree based on combined *psbA-trnH* and partial *petA-psbJ* sequences revealed four major groups of *Kaempferia* species. We suggest that the genus *Kaempferia* is a polyphyletic group, as *K. candida* was distantly related and did not group with other *Kaempferia* species. Polymorphic sites and indels of *psbA-trnH* and *petA-psbJ* can be used as DNA barcodes for species diagnosis of most *Kaempferia* and outgroup species. Nuclear DNA polymorphism should be examined to determine if there has been interspecific hybridization and chloroplast DNA introgression in these taxa.

Key words: *Kaempferia*; Chloroplast DNA; *psbA-trnH*; *petA-psbJ*; DNA barcode

INTRODUCTION

Kaempferia (Zingiberaceae) comprises about 60 species geographically distributed from India to Southeast Asia, where Thailand appears to be the richest biodiversity region with more than 20 extant species (Sirirugsa, 1992; Larsen and Saksuwan Larsen, 2006; Jenjittikul T and Larsen K, unpublished results). In Thailand, several *Kaempferia* species (i.e., *K. grandifolia*, *K. galanga*, *K. marginata*, *K. elegans*, and *K. roscoeana*) are well known for their ethnomedical uses by local people (Saensouk and Jenjittikul, 2001; Chuakul, 2003). *Kaempferia parviflora* (Krachai Dum) is famous as a health-promoting herb and is also used in several treatments such as dysentery, impotence, constriction, colic disorders, gastritis, etc. (Yenjai et al., 2003b, 2004).

Effective bioactive compounds have been isolated from several *Kaempferia* species. For instance, flavones (5-hydroxy-7-methoxyflavone and 5,7-dimethoxyflavone) from *K. parviflora* inhibited viral protease (Sookkongwaree et al., 2006). In addition, flavonoids (5,7,4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone) from *K. parviflora* and diterpenes (1,2,11-trihydroxypimara-8(14),15-diene and 1,11-dihydroxypimara-8(14),15-diene) from *K. marginata* exhibited anti-malarial activity against *Plasmodium falciparum* (Yenjai et al., 2004; Thongnest et al., 2005). Flavonoids (3,5,7,4'-tetramethoxyflavone and 5,7,4'-trimethoxyflavone) from *K. parviflora* and ethyl *p*-methoxycinnamate from *K. galanga* exhibited antimycobial activity against *Mycobacterium tuberculosis* and *Candida albicans* (Yenjai et al., 2003a,b, 2004). The ethanol extract of *K. galanga* exhibited anti-tumor promoter activity (Vimala et al., 1999). Therefore, plants in this genus are valuable sources of various bioactive compounds. In addition, *Kaempferia* are edible and valuable as ornamental plants and also used in cosmetic and perfume manufacturing (Ibrahim, 1999; Jenjittikul and Larsen, 2000; Saensouk and Jenjittikul, 2001).

Taxonomic identification of *Kaempferia* is difficult owing to the morphological similarity of vegetative parts among species and other genera in Zingiberaceae, such as *Boesenbergia*, *Cornukaempferia*, *Curcuma*, and *Scaphochlamys*. Without the floral parts, taxonomic identification to the species level is difficult. In addition, intraspecific variation causes more complicated problems in the classical taxonomy of this genus. *Kaempferia angustifolia*, for example, displays variations from narrow to broad leaves. The flowering season of *Kaempferia* is short, and inflorescences of some species (e.g., *K. candida*, *K. grandifolia* and *K. rotunda*) appear before leafy shoots and last only 1-2 weeks. In Thailand, most *Kaempferia* species are dormant during November to early May. Moreover, new species of *Kaempferia* have

been increasingly discovered. At least four new *Kaempferia* species found in Thailand have been recently discovered (Jenjittikul T and Larsen K, unpublished results). Cytological studies of 12 *Kaempferia* species in Thailand were reported and varied enormously in the number of chromosomes (2n = 22, 24, 33, 40, 44, and 55). Different ploidy levels were observed, e.g., in *K. rotunda* (2n = 22 or 33; Soontornchainaksaeng, 2005). The use of only vegetative morphological characters in the absence of reproductive parts and chromosome numbers is a major drawback for systematic and phylogenetic analyses and for authenticating morphologically similar *Kaempferia* species where no complete monograph is available at present (Saensouk and Jenjittikul, 2001).

Loesener (1930) taxonomically allocated 33 *Kaempferia* species into 5 subgenera. Of which, 9 species were found in Thailand and classified as members of subgenera *Soncorus* Horan. (i.e., *K. galanga*, *K. roscoeana*, *K. glauca*, *K. pulchra*, *K. laotica*, *K. elegans*, and *K. angustifolia*) and *Protanthium* Horan., including only precocious flowering species (i.e., *K. candida* and *K. rotunda*). However, more than 20 extant species have been recognized in Thailand to date.

Molecular systematics inferred from polymorphism of chloroplast (e.g., *matK*, *trnK*, *trnL-trnF*, and *psbA-trnH*) and nuclear (i.e., internal transcribed spacer, ITS) DNA sequences have gained acceptance in resolving taxonomic problems that have arisen from the traditional classification. Polymorphic DNA sequences of chloroplast *trnH-psbA* intergenic spacer and nuclear ribosomal ITS have shown the potential to be used as DNA barcodes for species identification in biodiversity studies of 99 plant species, representing 80 genera from 53 families (Kress et al., 2005b). In Zingiberaceae, the polymorphism of chloroplast *matK* gene and nuclear ITS sequences was used to examine the molecular phylogeny of *Alpinia* (Kress et al., 2005a). Nucleotide sequence polymorphism and indels of *psbA-trnH* and *petA-psbJ* spacers could be unambiguously applied as a molecular taxonomic key to authenticate 15 *Boesenbergia* species indigenous to Thailand. These data also supported further differentiation of *B. bambusetorum* from *B. longiflora* as a newly recognized *Boesenbergia* species (Techaprasan et al., 2006).

The genus *Kaempferia* is under revision with new species continually discovered (Larsen and Saksuwan Larsen, 2006). Little information of its phylogenetic history and molecular data inferred from chloroplast and/or nuclear sequences of *Kaempferia* has been reported. Recently, the phylogeny of Zingiberaceae was reported based on ITS and *matK* sequences, and *Kaempferia* is recognized as a monophyletic group (Kress et al., 2002). Nevertheless, only ITS sequences of 2 *Kaempferia* accessions and *matK* sequences of 4 *Kaempferia* taxa were included in the analyses. In this study, therefore, we applied a molecular approach in assessing the molecular systematics of *Kaempferia* found to be indigenous to Thailand using maternally inherited choloplast *psbA-trnH* and *petA-psbJ*. The phylogeny and sequence polymorphism for systematics and species identification of various *Kaempferia* are reported for the first time in this genus.

MATERIAL AND METHODS

Plant samples

Seventy-one accessions of *Kaempferia*, representing 15 recognized, 6 new, and 4 unidentified *Kaempferia* species were collected throughout Thailand. Fourteen accessions, representing 10 closely related Zingiberaceae species (*Boesenbergia rotunda*, *Gagnepainia godefroyi*, *G. thoreliana*, *Globba substrigosa*, *Scaphochlamys biloba*, *S. minutiflora*, *S. rubescens*, *Smithatris myanmarensis*, *S. supraneanae*, and *Stahlianthus* sp) were included as outgroups (Table 1).

Table 1. Species and geographic origins of *Kaempferia* and outgroup references used in this study.

Species	Sample No.	Geographic location	Voucher/ living specimen	GenBank accession No.		Remark
				psbA-trnH	*petA-psbJ*	
K. albomaculata sp nov. T. Jenjittikul & K. Larsen	1	Lop Buri, C	TT11560	GQ385994	GQ386077	
K. albomaculata sp nov. T. Jenjittikul & K. Larsen	2	Sukhothai, N	TT10225	GQ385995	GQ386078	
K. angustifolia Rosc.	1	Unknown	JT2005-13	GQ386041	GQ386124	cultivated specimen
K. angustifolia Rosc.	2	The Lao People's Democratic Republic	JT2007-42	GQ386042	GQ386125	
K. bambusetorum sp nov. K. Larsen & T. Jenjittikul	1	Phra Phutthabat, Saraburi, C	TT11559-1	GQ385998	GQ386081	brown-leaf
K. bambusetorum sp nov. K. Larsen & T. Jenjittikul	2	Phra Phutthabat, Saraburi, C	TT11559-2	GQ385999	GQ386082	green-leaf
K. candida Wall.	1	Thong Pha Phum, Kanchanaburi, SW	JT2007-7	GQ386003	GQ386086	
K. candida Wall.	2	Mae Sot, Tak, N	TT15730	GQ386004	GQ386087	
K. elegans (Wall.) Bak. in Hook. f.	1	Pang Sila Thong, Kamphaeng Phet, N	JT2007-40	GQ386005	GQ386088	
K. elegans (Wall.) Bak. in Hook. f.	2	Khlong Lan, Kamphaeng Phet, N	JT2007-34	GQ386006	GQ386089	
K. elegans (Wall.) Bak. in Hook. f.	3	Thi Lo Su, Tak, N	TT16410	GQ386009	GQ386092	
K. elegans (Wall.) Bak. in Hook. f.	4	Pa Khao Yai, Kanchanaburi, SW	JT2008-7	GQ386010	GQ386093	
K. elegans (Wall.) Bak. in Hook. f.	5	Thong Pha Phum, Kanchanaburi, SW	JT2007-8	GQ386011	GQ386094	
K. elegans (Wall.) Bak. in Hook. f.	6	Khao Kho, Phetchabun, NE	TT15690	GQ386007	GQ386090	
K. elegans (Wall.) Bak. in Hook. f.	7	Phop Phra, Tak, N	TT16450	GQ386008	GQ386091	
K. fallax Gagnep.	1	Sroi Sawan, Ubon Ratchathani, E	TT16539	GQ386018	GQ386101	
K. fallax Gagnep.	2 and 3	Pha Luang, Ubon Ratchathani, E	TT16670	GQ386019	GQ386102	
K. fallax Gagnep.	4	Pha Chana Dai, Ubon Ratchathani, E	TT16821	GQ386021	GQ386104	
K. filifolia K. Larsen	1	Pha Tam, Ubon Ratchathani, E	JT2007-23	GQ386022	GQ386105	
K. filifolia K. Larsen	2 and 3	Pha Luang, Ubon Ratchathani, E	TT16669	GQ386023	GQ386106	
K. galanga L.	1	Phop Phra, Tak, N	TT16452	GQ385979	GQ386062	cultivated specimen
K. galanga L.	2	Narathiwat, PEN	JT2008-12	GQ385977	GQ386060	cultivated specimen
K. galanga L.	3	The Lao People's Democratic Republic	TT s.n. 26June07	GQ385980	GQ386063	cultivated specimen
K. grandifolia S. Saensouk & T. Jenjittikul	1	Khon Kaen, NE	JT2007-3	GQ386017	GQ386100	
K. laotica Gagnep.	1	Sroi Sawan, Ubon Ratchathani, E	TT16535	GQ385987	GQ386070	
K. laotica Gagnep.	2	Pha Luang, Ubon Ratchathani, E	TT16688	GQ385985	GQ386068	
K. cf. *laotica* Gagnep.	3	Samet, Mueang, Buri Ram, E	JT2007-28	GQ385986	GQ386069	
K. larsenii P. Sirirugsa	1	Sroi Sawan, Ubon Ratchathani, E	TT16540	GQ385989	GQ386072	
K. larsenii P. Sirirugsa	2	Don Pho, Ubon Ratchathani, E	TT16657	GQ385991	GQ386074	
K. larsenii P. Sirirugsa	3	Pha Luang, Ubon Ratchathani, E	TT16683	GQ385992	GQ386075	
K. larsenii P. Sirirugsa	4	Si Mueang Mai, Ubon Ratchathani, E	TT16699	GQ385993	GQ386076	
K. larsenii P. Sirirugsa	5	Ubon Ratchathani, E	JT2006-5	GQ385990	GQ386073	
K. marginata Carey in Roscoe	1	Sangkha, Surin, E	TT16705	GQ385976	GQ386059	
K. marginata Carey in Roscoe	2	Khon Kaen, NE	JT2007-4	GQ385981	GQ386064	
K. marginata Carey in Roscoe	3	Prachin Buri, SE	TT15722	GQ385982	GQ386065	
K. marginata Carey in Roscoe	4	Sakon Nakhon, NE	TT15751	GQ385983	GQ386066	
K. marginata Carey in Roscoe	5	Saraburi, C	TT15721	GQ385984	GQ386067	
K. minuta sp nov. T. Jenjittikul & K. Larsen	1	Unknown	JT2005-4	GQ386046	GQ386129	
K. minuta sp nov. T. Jenjittikul & K. Larsen	2	Ubon Ratchathani, E	JT2007-33	GQ386045	GQ386128	
K. minuta sp nov. T. Jenjittikul & K. Larsen	3	Pa Dong Na Tam, Ubon Ratchathani, E	TT16550	GQ386043	GQ386126	
K. minuta sp nov. T. Jenjittikul & K. Larsen	4	Si Mueang Mai, Ubon Ratchathani, E	TT16694	GQ386044	GQ386127	
K. pardi sp nov. K. Larsen & T. Jenjittikul	1	Phetchabun, NE	JT2005-1	GQ385996	GQ386079	

Continued on next page

Table 1. (Continued)

Species	Sample No.	Geographic location	Voucher/ living specimen	GenBank accession No.		Remark
				psbA-trnH	*petA-psbJ*	
K. pardi sp nov. K. Larsen & T. Jenjittikul	2	Si Thep, Phetchabun, NE	JT2006-3	GQ385997	GQ386080	
K. parviflora Wall. ex Bak. in Hook. f.	1	Khao Kho, Phetchabun, NE	TT15691	GQ386012	GQ386095	cultivated specimen
K. parviflora Wall. ex Bak. in Hook. f.	2	Pa Khao Yai, Kanchanaburi, SW	JT2008-8	GQ386014	GQ386097	
K. parviflora Wall. ex Bak. in Hook. f.	3	Thong Pha Phum, Kanchanaburi, SW	JT2007-9	GQ386013	GQ386096	
K. parviflora Wall. ex Bak. in Hook. f.	4	Sai Yok, Kanchanaburi, SW	JT2007-17	GQ386015	GQ386098	
K. pulchra Ridl.	1	Kha On, Prachuap Khiri Khan, SW	JT2007-16	GQ386025	GQ386108	purple flowers
K. pulchra Ridl.	2	Khao Lsuang, Nakhon Si Thammarat, PEN	JT2006-7	GQ386026	GQ386109	purple flowers
K. pulchra Ridl.	3	Unknown	JT2005-11	GQ386027	GQ386110	purple flowers
K. pulchra Ridl.	4	Huai Yang, Prachuap Khiri Khan, SW	TT10144	GQ386028	GQ386111	white flowers
K. roscoeana Wall.	1	Sai Yok, Kanchanaburi, SW	JT2007-10	GQ386029	GQ386112	
K. roscoeana Wall.	2	Ta Mai Dang, Tak, N	TT16482	GQ386030	GQ386113	
K. rotunda L.	1	Ban Rai, Tak, N	JT2007-12	GQ386031	GQ386114	
K. rotunda L.	2	Doi Ruak, Tak, N	JT2007-13	GQ386032	GQ386115	
K. rotunda L.	3	Phop Phra,Tak, N	TT16456	GQ386037	GQ386120	
K. rotunda L.	4	Phu Khae, Saraburi, C	JT2006-4	GQ386033	GQ386116	
K. rotunda L.	5	Wiang Pa Pao, Chiang Rai, N	TT16385	GQ386035	GQ386118	
K. rotunda L.	6	Mae Sot, Tak, N	TT15732	GQ386034	GQ386117	
K. rotunda L.	7	Umphang, Tak, N	TT16426	GQ386036	GQ386119	
K. siamensis P. Sirirugsa	1	Phu Prao, Ubon Ratchathani, E	JT2007-25	GQ386039	GQ386122	
K. siamensis P. Sirirugsa	2	Sroi Sawan, Ubon Ratchathani, E	TT16534	GQ386040	GQ386123	
Kaempferia sp nov. 1 (Proh Mueang Kan)	1	Sai Yok, Kanchanaburi, SW	JT2007-11	GQ386001	GQ386084	
Kaempferia sp nov. 1 (Proh Mueang Kan)	2	Sai Yok, Kanchanaburi, SW	JT2007-18	GQ386002	GQ386085	
Kaempferia sp nov. 2 (Proh Mang Mum)	1	Pa Hua Khao Din, Tak, N	TT15793	GQ386038	GQ386121	
Kaempferia sp 1	1	Muak Lek, Saraburi, C	JT2006-1	GQ386000	GQ386083	
Kaempferia sp 2	1	Pha Tam, Ubon Ratchathani, E	JT2007-24	GQ385988	GQ386071	
Kaempferia sp 3	1	Khao Sam Chan, Kanchanaburi, SW	JT2007-21	GQ386016	GQ386099	
Kaempferia sp 4	1	Pha Luang, Ubon Ratchathani, E	TT16671	GQ385978	GQ386061	
Boesenbergia rotunda (L.) Mansf	1	Unknown	-	DQ408325	DQ104859	Techaprasan et al., 2006
Gagnepainia godefroyi (Baill.) K. Schum.	1	Sam Lan, Saraburi, C	JT2007-14	GQ386050	GQ386133	
G. godefroyi (Baill.) K. Schum.	2	Sroi Sawan, Ubon Ratchathani, E	TT16536	GQ386049	GQ386132	
Gagnepainia thoreliana (Baill.) K. Schum.	1	Thong Pha Phum, Kanchanaburi, SW	JT2007-6	GQ386051	GQ386134	
G. thoreliana (Baill.) K. Schum.	2	Phop Phra, Tak, N	TT16453	GQ386053	GQ386136	
Globba substrigosa King ex Bak. in Hook. f.	1	Pa Khao Yai, Kanchanaburi, SW	JT2008-6	GQ386048	GQ386131	
Scaphochlamys biloba (Ridl.) Holtt.	1	Su-ngai Padi, Narathiwat, PEN	JT2007-1	GQ386057	GQ386140	
Scaphochlamys minutiflora T. Jenjittikul & K. larsen	1	Su-ngai Padi, Narathiwat, PEN	JT2007-2	GQ386058	GQ386141	
Scaphochlamys rubescens T. Jenjittikul & K. Larsen	1	Narathiwat, PEN	-	DQ408335	DQ104878	Techaprasan et al., 2006
Smithatris myanmarensis W. J. Kress	1	Myanmar	TT16785	GQ386056	GQ386139	cultivated specimen
Smithatris supraneanae W. J. Kress & K. Larsen	1	Lop Buri, C	TT11561	GQ386055	GQ386138	
Stahlianthus sp	1	Unknown	JT2005-15	GQ386047	GQ386130	
Unknown sp 1	1	Klong Lan, Kamphaeng Phet, N	JT2007-37	GQ386052	GQ386135	
Unknown sp 2	1	Pa Khao Yai, Kanchanaburi, SW	JT2008-9	GQ386054	GQ386137	

N = Northern; NE = Northeastern; E = Eastern; SW = Southwestern; C = Central; SE = Southeastern; PEN = Peninsular Thailand; TT = Thaya (Tiptabiankarn) Jenjittikul; JT = Jiranan Techaprasan.

A map of Thailand illustrating sampling locations for replicate specimens of each *Kaempferia* species is shown in Figure 1. Voucher specimens were deposited at Suan Luang Rama IX herbarium, Thailand. The external morphology of some *Kaempferia* species in this study is shown in Figure 2.

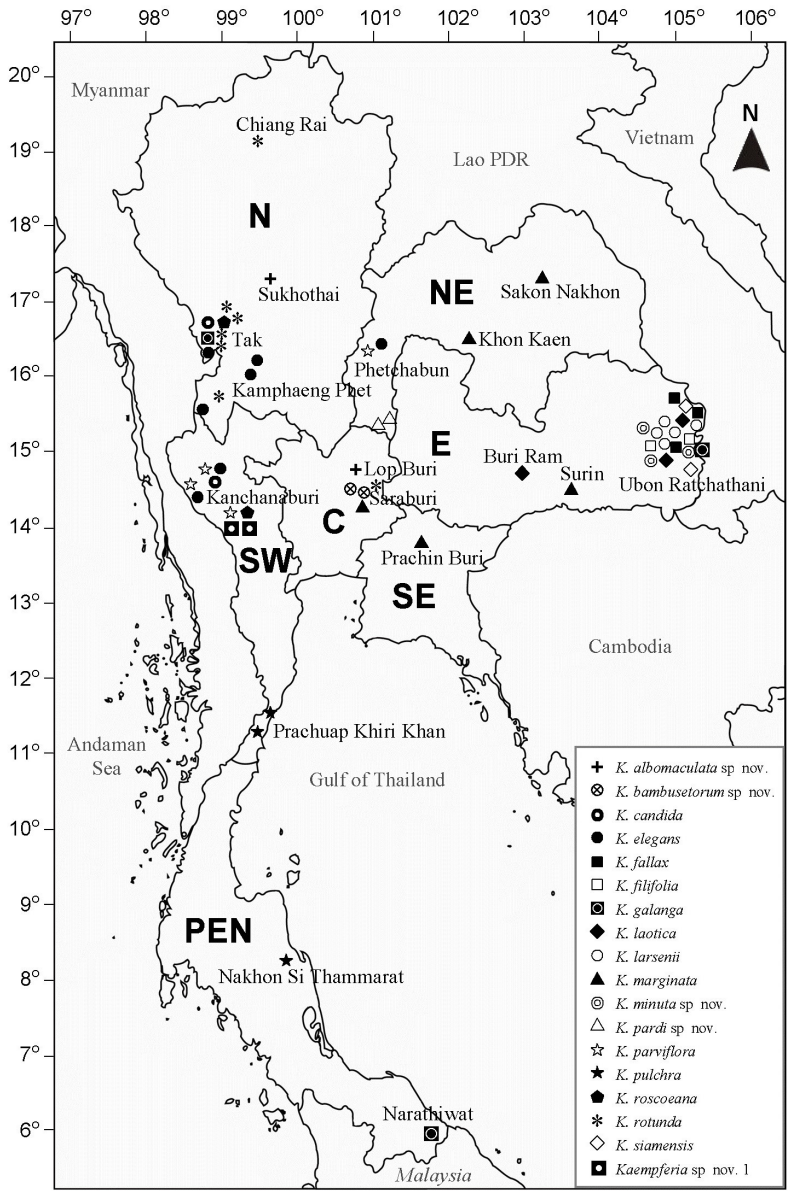

Figure 1. Map of Thailand showing multiple sample sites of various *Kaempferia* species. Solid lines indicate boundaries between floristic regions of Thailand. N = Northern; NE = Northeastern; E = Eastern; SW = Southwestern; C = Central; SE = Southeastern; PEN = Peninsular Thailand.

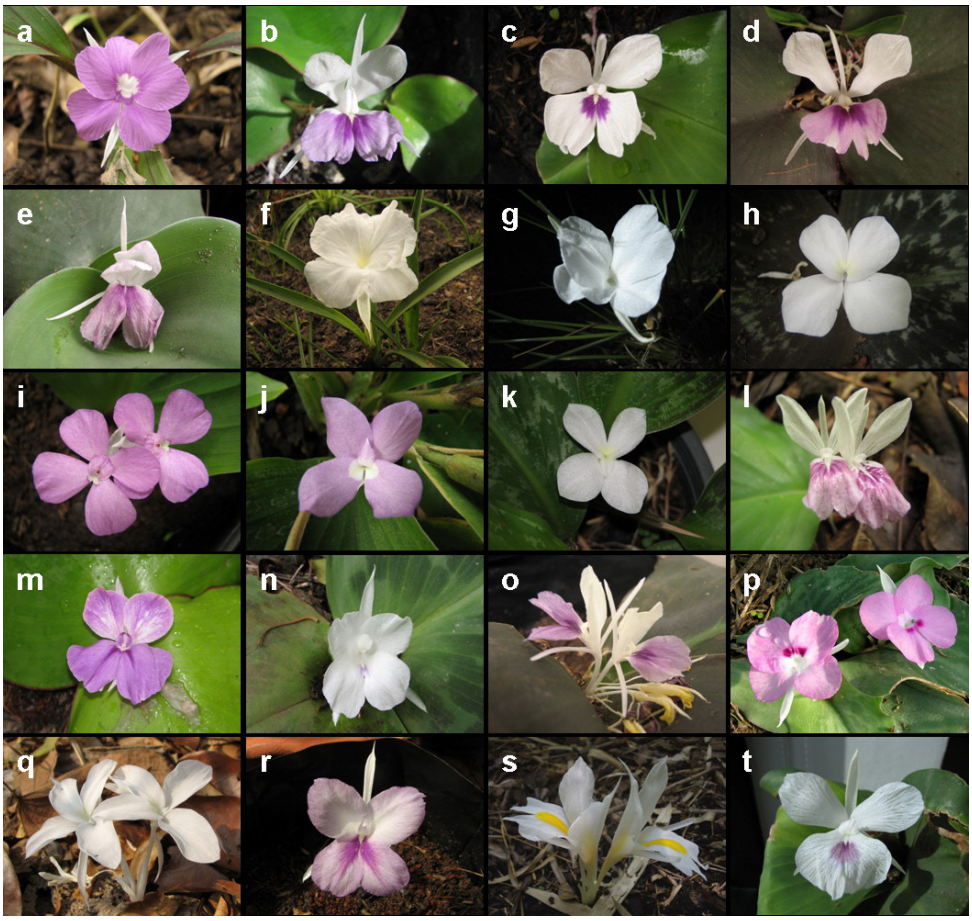

Figure 2. External morphology of some *Kaempferia* species. *K. larsenii* (a), *K. laotica* (b), *K. galanga* (c), *K. marginata* (d), *K. siamensis* (e), *K. fallax* (f), *K. filifolia* (g), *K. roscoeana* (h), *K. elegans* (i), *K. pulchra* (purple flower, j), *K. pulchra* (white flower, k), *K. parviflora* (l), *K. albomaculata* sp nov. (m), *K. pardi* sp nov. (n), *K. bambusetorum* sp nov. (o), *K. minuta* sp nov. (p), *K. grandifolia* (q), *K. rotunda* (r), *K. candida* (s), and *Kaempferia* sp 3 (t).

DNA extraction, polymerase chain reaction, and DNA sequencing

Genomic DNA was extracted from fresh young leaves or flowers of each plant using a modification of the CTAB method of Doyle and Doyle (1987). The *psbA-trnH* and *petA-psbJ-psbL* regions of each taxon were separately amplified in a 50-µL reaction volume containing 1X buffer, $MgCl_2$ (1.5 and 3.0 mM for *psbA-trnH* and *petA-psbJ-psbL*, respectively), dNTPs (0.20 and 0.24 µM), primers (0.20 µM each of *psbA*-1F: 5′-CTTGGTATGGAAGTAATGCA-3′ and *trnH*-1R: 5′-ATCCACTTGGCTACATCCG-3′, and 0.24 µM each of *petA*-F: 5′-AGGTT CAATTGTMCGAAATG-3′ and *psbL*-R: 5′-GTACTTGCTGTTTTATTTTC-3′), 200-400 ng total DNA and 1 U *Taq* DNA polymerase (Techaprasan et al., 2006). Polymerase chain reaction was carried out consisting of an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55° or 58°C for 1 min, and extension

at 72°C for 30 s or 1 min for the respective regions. The final extension was carried out for 7 min at 72°C. The amplified *psbA-trnH* and *petA-psbJ-psbL* were direct-sequenced for both directions on an automated DNA sequencer using the original amplification primer as the sequencing primer.

Data analysis

Sequences of *psbA-trnH* and *petA-psbJ-psbL* spacers were aligned using ClustalW incorporated in BioEdit version 7.0.5.2 (Hall, 1999) and further edited manually. Nucleotide sequence divergence between pairs of taxa was calculated using a Kimura (1980) 2-parameter model without indel consideration. Maximum parsimony analysis was carried out using Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0 Beta 10 (Swofford, 2004). Gaps were treated as missing data. To reconstruct the most parsimonious trees, a heuristic search was executed using random (1000 replicates) stepwise addition followed by tree bisection-reconnection branch swapping. Bootstrapping (1000 replicates) was performed with random sequence addition. Bootstrapping values (>50%) were superimposed on the strict consensus tree to illustrate confidence level of relationships among samples investigated in this study.

RESULTS

The amplified *psbA-trnH* and *petA-psbJ-psbL* spacers in *Kaempferia* were approximately 800 and 1200 bp in length with the exception of 400 and 900 bp for *S. biloba* and 800 and 900 bp for *S. minuta*, respectively. Nucleotide sequences at the 3′ end of *petA-psbJ-psbL* spacers were missing and not included in the analyses. Sequences of *psbA-trnH* and partial *petA-psbJ* of all investigated specimens in this study were deposited in GenBank with accession Nos. GQ385976-386058 and GQ386059-386141, respectively.

Within *Kaempferia*, nucleotide sequences of *psbA-trnH* ranged between 714 bp (*K. elegans* JT2007-8 and *K. parviflora* JT2007-17) and 798 bp (*K. filifolia* JT2007-23) and were 1010 bp after multiple sequence alignments. Likewise, those of the partial *petA-psbJ* ranged between 659 bp (*K. pulchra* TT10144 and *K. roscoeana* TT16482) and 754 bp (*K. rotunda* TT15732) in length and resulted in the multiple alignments of 865 bp. For the combined data, 1875 bp in length were obtained, including 126 variable parsimoniously uninformative sites and 116 parsimoniously informative characters (Table 2), indicating that both chloroplast DNA spacers provided limited phylogenetic information. Pairwise nucleotide divergence of *psbA-trnH* within *Kaempferia* and across all examined taxa ranged between 0.00-3.31 and 0.00-3.33% (*K. elegans* TT16410 and *G. thoreliana*), while that of the partial *petA-psbJ* was between 0.00-2.03 and 0.00-4.69% (*K. fallax* TT16670 and *S. rubescens*), respectively.

The strict consensus tree generated from the combined data of the spacers possessed 321 informative mutation steps with consistency and retention indices of 0.82 and 0.91, respectively (Figure 3). Two accessions of *K. candida* were separated from other *Kaempferia* members. Disregarding *K. candida*, *K. pulchra*, and *K. roscoeana*, other *Kaempferia* species were separated into 4 major clades: clade A (*K. marginata*, *K. galanga*, *K. laotica*, *K. larsenii*, *K. albomaculata* sp nov., *K. pardi* sp nov., and *K. bambusetorum* sp nov.); clade B includes only the Northeastern species (*K. minuta* sp nov., *K. angustifolia*, *K. fallax*, *K. filifolia*, and *K. siamensis*); clade C includes *Kaempferia* species whose inflorescences appear before leafy shoots, i.e., *K. rotunda*, *K. grandifolia*, *Kaempferia* sp 1, *Kaempferia* sp nov. 1, and *Kaempferia* sp nov. 2; clade D (*K. elegans* and *K. parviflora*).

Table 2. Sequence characteristics and nucleotide sequence divergence of *psbA-trnH* and *petA-psbJ* across *Kaempferia* species and outgroup references.

	psbA-trnH[a]	*petA-psbJ*[b]	Combined data
Number of nucleotides	714-798	659-754	1875
Number of uninformative-variable sites	61	65	126
Number of informative characters	68	48	116
Percentage of interspecific sequence divergence within *Kaempferia*[c]	0.00-3.31	0.00-2.03	0.00-2.11
Percentage of interspecific and intergeneric sequence divergence[c]	0.00-3.33	0.00-4.69	0.00-2.61
Percentage of intraspecific sequence divergence[c]			
K. albomaculata sp nov.	0.00	0.00	0.00
K. angustifolia	0.00	0.00	0.00
K. bambusetorum sp nov.	0.00	0.00	0.00
K. candida	0.00	0.00	0.00
K. galanga	0.00	0.00	0.00
K. laotica	0.00	0.00	0.00
K. minuta sp nov.	0.00	0.00	0.00
K. pardi sp nov.	0.00	0.00	0.00
Kaempferia sp nov. 1	0.00	0.00	0.00
K. fallax	0.00-0.27	0.00-0.14	0.00-0.14
K. filifolia	0.00	0.00-0.30	0.00-0.14
K. elegans	0.00-0.98	0.00-0.81	0.00-0.77
K. larsenii	0.00	0.00-0.27	0.00-0.14
K. marginata	0.00-0.41	0.00-0.15	0.00-0.28
K. parviflora	0.14-1.15	0.45-0.76	0.22-0.85
K. pulchra	0.00-0.83	0.15-0.79	0.07-0.59
K. roscoeana	0.00	0.61	0.29
K. rotunda	0.00-0.82	0.14-0.84	0.14-0.83
K. siamensis	0.13	0.15	0.14
G. godefroyi	0.56	0.70	0.63
G. thoreliana	0.00	0.00	0.00

GenBank accession Nos. [a]GQ385976-386058 and [b]GQ386059-386141; [c]indels were not included in the analysis.

Like *K. roscoeana*, geographically different samples of *Kaempferia* species in clade B (except *K. filifolia*) clustered together. However, evolutionary relationships of other clades were not fully resolved, especially with *K. elegans* and *K. parviflora*, members of clade D. Within clade C, *K. rotunda* from different locations showed intraspecific sequence polymorphism and clustered separately with other taxa (*Kaempferia* sp 1, *Kaempferia* sp nov. 2, and *K. grandifolia*). However, two *Kaempferia* sp nov. 1 accessions grouped together (90% bootstrapping value). Members of clade A showed less informative sequence variation, resulting in unresolved evolutionary relationships. For example, *K. galanga*, *K. marginata* (except JT2007-4 and TT15721), and *Kaempferia* sp 4 possessed identical sequences. Moreover, only one indel could differentiate *K. larsenii* (except TT16540) and *K. laotica*.

No intraspecific sequence variation was observed in *K. candida*, *K. angustifolia*, *K. laotica*, *K. galanga*, *K. pardi* sp nov., *K. bambusetorum* sp nov., *K. albomaculata* sp nov., *K. minuta* sp nov., *Kaempferia* sp nov. 1, and *G. thoreliana*. In contrast, intraspecific sequence polymorphism was observed in different populations of *K. fallax*, *K. filifolia*, *K. elegans*, *K. pulchra*, *K. rotunda*, *K. marginata*, *K. parviflora*, *K. larsenii*, *K. roscoeana*, *K. siamensis*, and *G. godefroyi*.

Although phylogenetically unresolved relationships of some *Kaempferia* were observed, polymorphic sites and indels of *psbA-trnH* and *petA-psbJ* can be used for species authentication of most *Kaempferia* species, i.e., *K. laotica*, *K. angustifolia*, *K. siamensis*, *K. grandifolia*, *K. roscoeana*, *K. candida*, *K. pardi* sp nov., *K. bambusetorum* sp nov., *K. albomaculata* sp nov., *K. minuta* sp nov., and *Kaempferia* sp nov. 1 and outgroups (Figures 4 and 5). Moreover, a string of sequences in *psbA-trnH* (AGTTTAGATATTT) and *petA-psbJ* (CTACAA) could differentiate

members of subgroup A1 (*K. albomaculata* sp nov./*K. larsenii*/*K. laotica*) and those of clade A from other taxa, respectively. In addition, species-specific sequences in *psbA-trnH* for *K. albomaculata* sp nov. (AGAT, G and TATATAAATATATAAA), *K. bambusetorum* (GTATATTAAATACAAAT) and *K. laotica*/*K. larsenii* (CATAA) were also found.

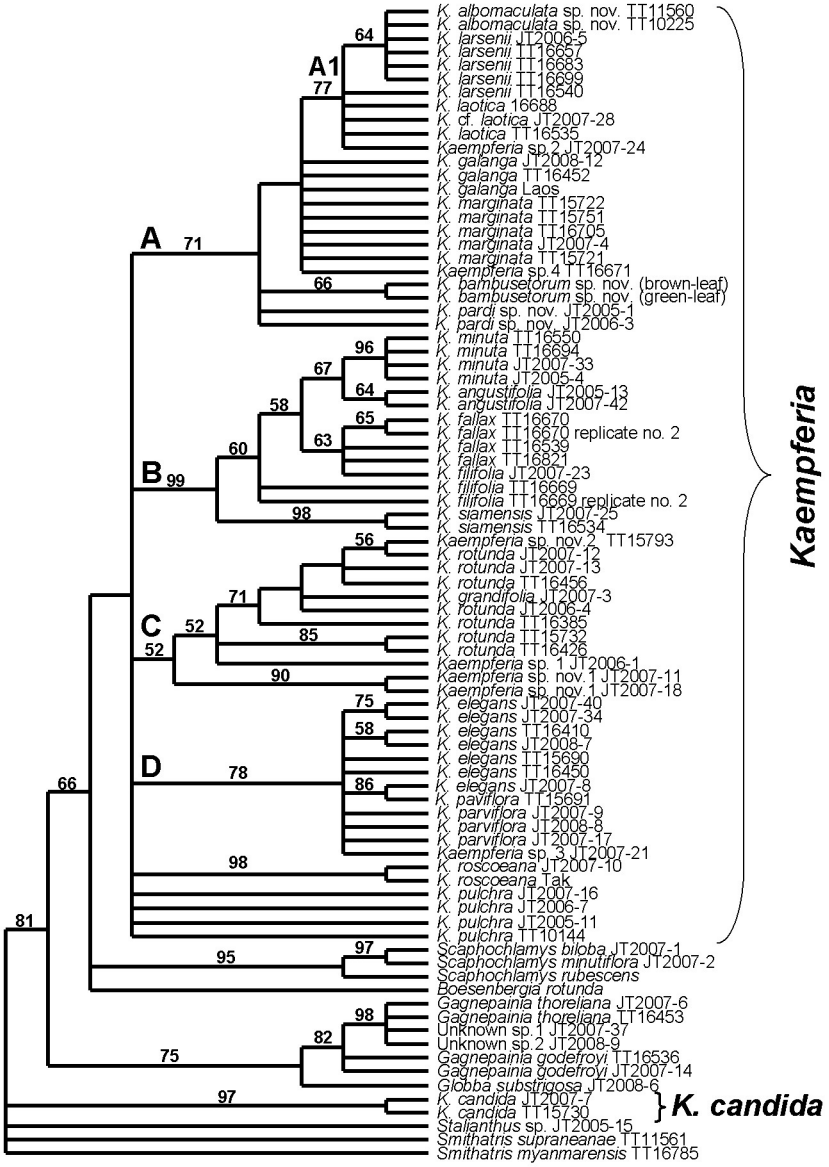

Figure 3. A strict consensus tree constructed from 778,100 of the most parsimonious trees based on combined *psbA-trnH* and *petA-psbJ* sequences (tree length = 321 steps; consistency index = 0.82 and retention index = 0.91). Values at the node (>50%) represent the percentage of times that the particular node occurred in 1000 replicates generated by bootstrapping the original sequences.

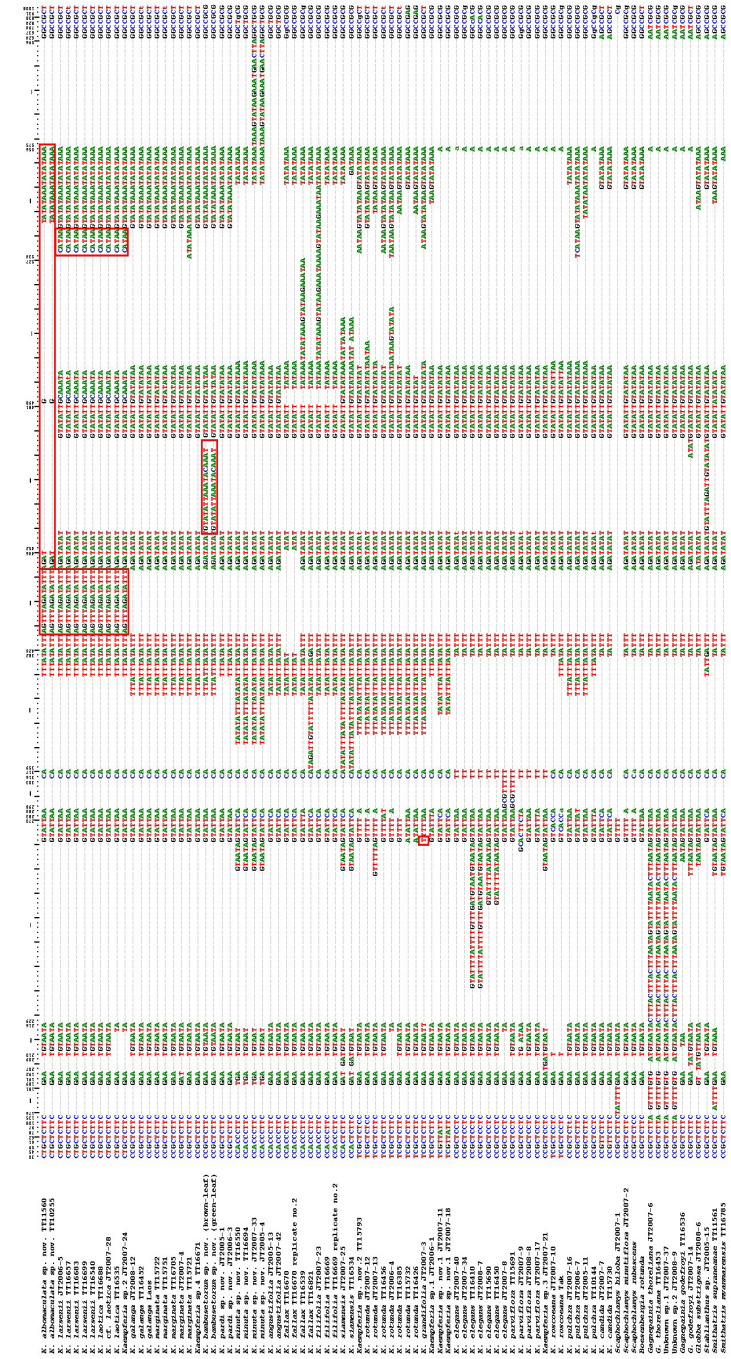

Figure 4. Polymorphic sites and indels of *psbA-trnH* sequences used for authenticating most *Kaempferia* and outgroup species. Boxes indicate strings of sequences that are able to distinguish members of subgroup A1 and *K. albomaculata*, *K. bambusetorum*, *K. laotica*/*K. larsenii*, and *K. grandifolia* from other taxa.

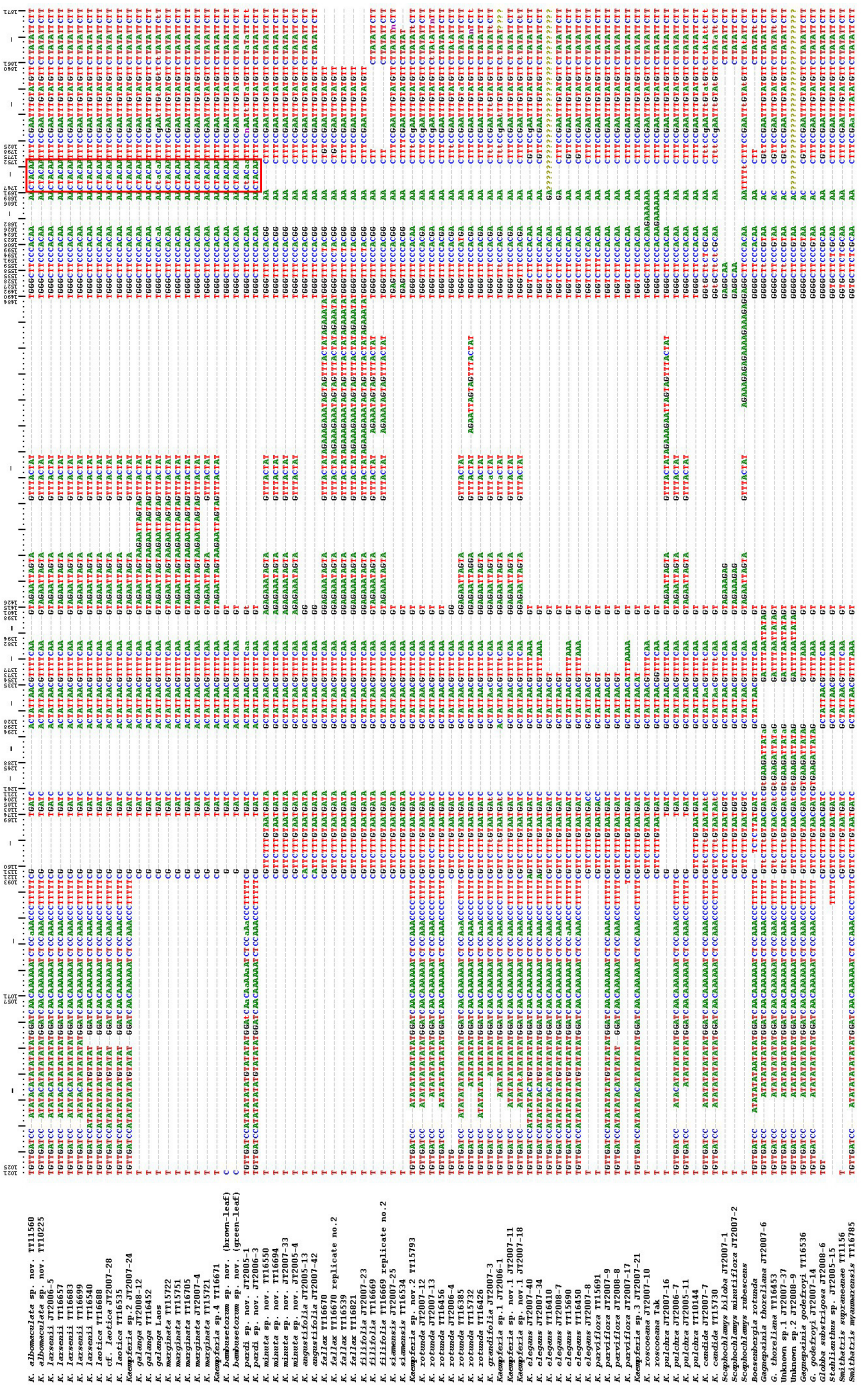

Figure 5. Polymorphic sites and indels of *petA-psbJ* sequences used for authenticating most *Kaempferia* and outgroup species. Boxes indicate strings of sequences that are able to distinguish members of clade A from other taxa.

DISCUSSION

Like most genera (e.g., *Globba*, *Curcuma*, *Alpinia*, *Boesenbergia*, etc.) in Zingiberaceae, taxonomic difficulties were also observed in *Kaempferia*. In *Boesenbergia*, nucleotide polymorphisms and indels of *psbA-trnH* and *petA-psbJ* were successfully applied for species authentication, even though limited sequence divergence was observed (0.00-3.53 and 0.00-2.53%, respectively; Techaprasan et al., 2006). Similarly, limited sequence divergence of *psbA-trnH* and the partial *petA-psbJ* spacers in *Kaempferia* and outgroups (0.00-3.33 and 0.00-4.69%, respectively) was also observed in this study.

Phylogenetic reconstruction based on the parsimony approach was carried out, but indels of the multiple aligned *psbA-trnH* and *petA-psbJ* sequences were excluded from the analysis as they caused low bootstrapping values in several other branches and more unresolved evolutionary relationships of the reconstructed tree (data not shown).

Previously, *Kaempferia* was regarded as a monophyletic genus (Kress et al., 2002). Based on our study on the maternally inherited chloroplast (cpDNA) data, *K. candida* was clearly allocated as the well-isolated clade outside *Kaempferia* members. The results suggest that *K. candida* may be misallocated to be a member of this genus. Nevertheless, we cannot rule out the possible consequence of reticulate evolution (e.g., intergeneric hybridization and introgression) in this species. Therefore, the taxonomic status of *K. candida* should be further examined by multiple loci of biparentally segregrated nuclear DNA markers to elucidate this speculation.

Phylogenetic analysis clearly revealed 4 different clades of *Kaempferia* in this study. Clade A consisted of complex species (e.g., *K. marginata*, *K. galanga*, and *K. laotica*). Of these, *K. galanga* is a cultivated species and believed to have been introduced from India (Holttum, 1950; Larsen and Saksuwan Larsen, 2006), whereas *K. marginata* is naturally distributed locally. Taxonomic key to *Kaempferia* species of Thailand described that leaf margin and labellum of *K. marginata* is purple, whereas leaf margin of *K. galanga* is usually white and its labellum is white with purple marking at the base (Sirirugsa, 1992). However, labellums of *K. marginata* display color variation from white to purple. Nucleotide sequences of *psbA-trnH* and *petA-psbJ* of *K. galanga* and *K. marginata* (excluding JT2007-4 and TT15721, which showed intraspecific polymorphism) were identical, suggesting that they are recently matriarchally diverse. More samples of *K. marginata* and *K. galanga* from the original resources (i.e., India) should be included in the analysis to elucidate whether *K. galanga* is a cultivated variant of *K. marginata*.

Within clade B, *K. filifolia* JT2007-23 clustered with *K. fallax*. Flowers of *K. fallax* and *K. filifolia* are similar but leaves of *K. fallax* are elliptic-linear to linear, whereas those of *K. filifolia* are filiform (Sirirugsa, 1992). Nucleotide polymorphism and indels of *psbA-trnH* and *petA-psbJ* of *K. filifolia* from Pha Luang (TT16669 and TT16669 replicate No. 2) were obviously different from *K. fallax* from Sroi Sawan (TT16539), Pha Luang (TT16670 and TT16670 replicate No. 2), and Pha Chana Dai (TT16821), whereas those of *K. filifolia* from Pha Tam (JT2007-23) were similar to those of *K. fallax*. However, ITS sequences of these taxa are identical (data not shown). In addition, a phylogenetic tree inferred from amplified fragment length polymorphism (AFLP) analysis confirmed their close relationships (Techaprasan J, unpublished results). Accordingly, *K. fallax* and *K. filifolia* should be regarded as sibling species. *K. siamensis*, *K. minuta* sp nov. and *K. angustifolia* did not form a species complex and phylogenetically recognized as separate species.

Loesener (1930) included three *Kaempferia* species in the subgenus *Protanthium* Horan. (*K. rotunda*, *K. candida*, and *K. fissa*) in the previous classification of Zingiberaceae. Based on cytological, anatomical and morphological studies, *K. grandifolia* was recently recognized as a new *Kaempferia* endemically found in Thailand. It exhibits the closest morphology to *K. roscoeana* and is classified as a member of the subgenus *Soncorus* Horan., but its inflorescences appear before leafy shoots, as seen in *K. rotunda* (Saensouk and Jenjittikul, 2001). In this study, *K. grandifolia* was phylogenetically placed between *K. rotunda* varieties. One polymorphic site (T at position 266) in *psbA-trnH* could distinguish *K. grandifolia* from other species. Accordingly, we argue that *K. grandifolia* should be evolutionarily descended from the *K. rotunda* lineage.

There has been a controversy over the species status of *K. elegans* and *K. pulchra* (Holttum, 1950; Smith, 1987). Searle (1999) regarded *K. pulchra* Ridl. as a synonym of *K. elegans* Wall. However, several authors recognized these plants as different species (Sirirugsa, 1992; Larsen and Saksuwan Larsen, 2006). The anther crests of *K. pulchra* are clawed-blade oblanceolate (leaf-shape) whereas those of *K. elegans* are orbicular (circular or nearly so; Sirirugsa, 1992). Our results revealed phylogenetic separation of *K. pulchra* from other *Kaempferia* species. Therefore, *K. pulchra* and *K. elegans* should be systematically recognized as different species.

The tree topology of *Kaempferia* in this study also indicated that *K. parviflora* and *K. elegans* were closely related phylogenetically, and 2 samples (*K. parviflora* TT15691 and *K. elegans* JT2007-8) may be their interspecific hybrid. More suitable molecular markers (e.g., polymorphic nuclear DNA and AFLP-derived markers) should be tested to identify whether introgression of cpDNA between *K. parviflora* TT15691 and *K. elegans* JT2007-8 as a consequence of hybridization readily occurs.

Generally, sequence polymorphism of *psbA-trnH* and *petA-psbJ* can be applied to DNA barcoding in *Kaempferia*. However, only one indel at the 5′ end of *petA-psbJ* was able to further differentiate *K. laotica* and *K. larsenii* (excluding TT16540). This indel seems to be a part of the inverted repeat of the stem-loop or hairpin structures commonly found in angiosperms (Kim and Lee, 2004), including Zingiberales (Swangpol et al., 2007). However, external morphology of leaves can be used to unambiguously differentiate *K. larsenii* and *K. laotica*. Leaves of *K. larsenii* are smaller (0.5-1 x 6-9 cm), elliptic-linear and erect, whereas those of *K. laotica* are obviously larger (7-10.5 x 7-12.5 cm), suborbicular and horizontal close to the ground (Sirirugsa, 1992).

The 46-63-bp indels of the stem-loop structures at the 5′ end of *petA-psbJ* sequences were also observed in half of the *Kaempferia* investigated (e.g., *K. laotica*, *K. larsenii*, *K. albomaculata* sp nov., *K. pardi* sp nov., *Kaempferia* sp 1, *Kaempferia* sp nov. 1, *Kaempferia* sp nov. 2, some accessions of *K. elegans*, *K. parviflora*, *K. pulchra*, and *K. rotunda*) and 4 outgroups (*B. rotunda*, *G. godefroyi*, *G. thoreliana*, and *S. myanmarensis*) species. However, this DNA region may not be appropriate to either include in phylogenetic reconstruction or to use for DNA barcoding, owing to the possibility of being phylogenetically and systematically misleading (Kelchner and Wendel, 1996). For example, 6 accessions of *K. elegans* showed 4 different types of this indel, but it was absent in *K. elegans* JT2007-8.

Intraspecific sequence divergences in different populations of *K. elegans* (0.00-0.77%), *K. parviflora* (0.22-0.85%), *K. pulchra* (0.07-0.59%), and *K. rotunda* (0.14-0.83%) were greater than in other *Kaempferia* species indicating that these species were highly diverse. Therefore, *psbA-trnH* and *petA-psbJ* spacers could not be used as DNA barcodes in *K. elegans*, *K. parviflora*, *K. pulchra*, *K. ro-*

tunda, some *K. larsenii*, between *K. marginata* and *K. galanga*, and between *K. fallax* and *K. filifolia*.

Apart from that, polymorphic indels and nucleotides of *psbA-trnH* and *petA-psbJ* could be used for species authentication of most *Kaempferia* species (e.g., *K. angustifolia*, *K. candida*, *K. laotica*, *K. roscoeana*, *K. siamensis*, *K. albomaculata* sp nov., *K. bambusetorum* sp nov., *K. minuta* sp nov., *K. pardi* sp nov., and *Kaempferia* sp nov. 1) and outgroups (e.g., *G. godefroyi* and *G. thoreliana*) for which more than one specimen was available for each species.

Our results based on maternally inherited cpDNA data reveal that *Kaempferia* sp 1 should be recognized as newly unidentified *Kaempferia* species, and that *Kaempferia* sp 2, 3, and 4 are not new species, exhibiting identical *psbA-trnH* and *petA-psbJ* sequences to *K. laotica*, *K.* cf. *parviflora*, and the *K. marginata*/*K. galanga* species complex, respectively. Phylogenetic analysis also confirmed the species status of 4 newly described *Kaempferia* species (*K. pardi* sp nov., *K. bambusetorum* sp nov., *K. albomaculata* sp nov., and *K. minuta* sp nov.) and *Kaempferia* sp nov. 1. Although the floral parts of *Kaempferia* sp nov. 2 (TT15793) were clearly different from *K. rotunda*, nucleotide polymorphism and indels of *psbA-trnH* and *petA-psbJ* were not sufficiently informative to verify that it is not a morphological variant of *K. rotunda* but a new *Kaempferia* species.

Apparently, *psbA-trnH* and *petA-psbJ* polymorphism unambiguously authenticated unknown species 1 and 2 as *G. thoreliana*. The inflorescence of the unknown sp 1 bloomed after collection for approximately one year, and it was concordantly classified as *G. thoreliana* on a morphological basis. Moreover, *psbA-trnH* and *petA-psbJ* sequences clearly suggest that the JT2007-28 specimen, which was initially misidentified as *K. marginata*, should be *K.* cf. *laotica*. This further confirms that *psbA-trnH* and *petA-psbJ* spacers are potentially useful as DNA barcodes in most *Kaempferia* species found to be indigenous in Thailand.

Previously, Techaprasan et al. (2006) examined the sequence polymorphism of 22 *Boesenbergia* taxa at 3 cpDNA regions (*matK*, *psbA-trnH*, and *petA-psbJ*), and all taxa were unambiguously differentiated. In this study, polymorphism of *psbA-trnH*, and *petA-psbJ* in 71 *Kaempferia* and 14 outgroup taxa were examined. Informative characters of *psbA-trnH* and *petA-psbJ* sequences were sufficient to phylogenetically differentiate several taxa. Nucleotide polymorphism and indels of their sequences provided strong phylogenetic signals and could be applied in authenticating most *Kaempferia* and their closely related species. To confirm taxonomic status of problematic *Kaempferia* species (e.g., *K. candida*) and to clarify evolutionary relationships of this genus, a global sampling of *Kaempferia* and an examination by both biparentally inherited nuclear DNA (e.g., ribosomal ITS and polymorphic AFLP-derived sequences) and these (*psbA-trnH* and *petA-psbJ*) or other cpDNA regions are required for studying the molecular systematics of the whole genus *Kaempferia*.

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