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Genetic Variation and Species Identification of Thai *Boesenbergia* (Zingiberaceae) Analyzed by Chloroplast DNA Polymorphism

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Genetic variation and molecular phylogeny of 22 taxa representing 14 extant species and 3 unidentified taxa of *Boesenbergia* in Thailand and four outgroup species (*Cornukaempferia aurantiflora*, *Hedychium biflorum*, *Kaempferia parviflora*, and *Scaphochlamys rubescens*) were examined by sequencing of 3 chloroplast (cp) DNA regions (*matK*, *psbA-trnH* and *petA-psbJ*). Low interspecific genetic divergence (0.25-1.74%) were observed in these investigated taxa. The 50% majority-rule consensus tree constructed from combined chloroplast DNA sequences allocated *Boesenbergia* in this study into 3 different groups. Using *psbA*-1F/*psbA*-3R primers, an insertion of 491 bp was observed in *B. petiolata*. Restriction analysis of the amplicon (380-410 bp) from the remaining species with *Rsa* I further differentiated *Boesenbergia* to 2 groupings; I (*B. basispicata*, *B. longiflora*, *B. longipes*, *B. plicata*, *B. pulcherrima*, *B. tenuispicata*, *B. thorelii*, *B. xiphostachya*, *Boesenbergia* sp.1 and *Boesenbergia* sp.3; phylogenetic clade A) that possesses a *Rsa* I restriction site and II (*B. curtisii*, *B. regalis*, *B. rotunda* and *Boesenbergia* sp.2; phylogenetic clade B and *B. siamensis*; phylogenetic clade C) that lacks a restriction site of *Rsa* I. Single nucleotide polymorphism (SNP) and indels found can be unambiguously applied to authenticate specie-origin of all investigated samples and revealed that *Boesenbergia* sp.1, *Boesenbergia* sp.2 and *B. pulcherrima* (Mahidol University, Kanchanaburi), *B.* cf. *pulcherrima*1 (Prachuap Khiri Khan) and *B.* cf. *pulcherrima*2 (Thong Pha Phum, Kanchanaburi) are *B. plicata*, *B. rotunda* and *B. pulcherrima*, respectively. In addition, molecular data also suggested that *Boesenbergia* sp.3 should be further differentiated from *B. longiflora* and regarded as a newly unidentified *Boesenbergia* species.

Keywords: *Boesenbergia*, Chloroplast DNA, DNA sequencing, Genetic variation, PCR-RFLP, Species identification

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Introduction

Boesenbergia, a member of Zingiberaceae, are composed of approximately 80 species worldwide and 19 of which are indigenous to Thailand (Saensouk and Larsen, 2001). Only *B. rotunda* is cultivated commercially and its rhizomes have been used for medicinal (i.e. treatment of colic disorder and as an aphrodisiac in folk medicine; Trakoontivakorn *et al*., 2001) and culinary purposes. The rhizomes of this species contain active constituents against HIV-1 protease (Tewtrakul *et al*., 2003) and those exhibiting anti-tumor, anti-mutagenic and anti-inflammatory activities (Murakami *et al*., 1994; Trakoontivakorn *et al*., 2001; Tuchinda *et al*., 2002). Due to these properties, *Boesenbergia* species has gained attention as important sources of active constituents for medicinal treatment.

Reproductive parts (e.g. anther crests, labellum and inflorescence position) play an important role for taxonomy of *Boesenbergia*. Therefore, taxonomic identification of these species based primarily on morphological characters without reproductive parts, is not sufficient for species identification. However, *Boesenbergia* possesses the dormancy period from the end of December to the middle of May. The flowering season of *Boesenbergia* is short, and some species have similar floral morphology, but differ in colors and inflorescence positions.

One of our purposes is to identify and characterize

metabolic profiles of *Boesenbergia* species where no data are available at present. Nevertheless, *Boesenbergia* display systematic problems owing to morphological variation at the intraspecific level. For instance, *B. curtisii* can possess black or white leaf sheaths and *B. plicata* can have yellow or red flowers (Vanijajiva *et al*., 2003). Typically, external characteristics are influenced by a variety of habitats and environmental conditions. Accordingly, two sympatric species may be morphologically similar and misidentified as a single species. Conversely, allopatric taxa in different habitats may show ecomorphological variation and have questionable species status. Authentication of *Boesenbergia* in Thailand by molecular markers is thus required to unambiguously identify the correct species of different forms of plant materials.

The basic information on genetic diversity and systematics of *Boesenbergia* is rather limited. Vanijajiva *et al*. (2003) examined 11 taxa of *Boesenbergia*, 6 taxa of *Kaempferia* and 2 taxa of *Scaphochlamys* using isozyme analysis. Peroxidase (POX), superoxide dismutase (SOD), glutamate dehydrogenase (GDH) and malate dehydrogenase (MDH) yielded polymorphic patterns across investigated taxa. A UPGMA dendrogram and a principal component analysis showed closer intergeneric relationships between *Boesenbergia* and *Scaphochlamys* than *Kaempferia*. Results were subsequently confirmed by randomly amplified polymorphic DNA (RAPD) analysis (Vanijajiva *et al*., 2005).

Polymorphism of chloroplast DNA (cpDNA), especially *trnK*, *matK*, and an intergenic *trnL-trnF*, has been commonly used for phylogenetic studies of various plants (Shaw *et al*., 2005). The *matK* which is the most rapidly evolving coding region in the chloroplast genome, has successfully been used for phylogenetic analysis at lower taxonomic levels in *Saxifraga* (Soltis *et al*., 1996) and *Chrysosplenium* (Soltis *et al*., 2001), Saxifragaceae, *Paeonia*, Paeoniacae (Sang *et al*., 1997), *Linanthus*, Polemoniaceae (Bell and Patterson, 2000), *Lycium*, Solanaceae (Fuguda *et al*., 2001), *Clintonia*, Liliaceae (Hayashi *et al*., 2001), *Coelogyne*, Orchidaceae (Gravendeel *et al*., 2001), *Myosotis*, Boraginaceae (Winkworth *et al*., 2002), *Hordeum*, Poaceae (Nishikawa *et al*., 2002), and *Hypochaeris*, Asteracae (Samuel *et al*., 2003). Within Zingiberaceae, *trnK* was used for identification of *Curcuma* species (Cao *et al*., 2001) while *trnK* (including *matK*) in combination with nuclear internal transcribed spacer (ITS) were used to study triploid formation in *Globba* (Takano and Okada, 2002). Recently, *matK* was used to examine phylogenetic relationships of Zingiberaceae (Kress *et al*., 2002) and weakly supported a monophyly of *Boesenbergia* (54% bootstrap value).

More recently, intergenic spacers (i.e. *psbA-trnH* and *petA-psbJ*) have been increasingly used for studies of genetic variation and phylogenetic analysis due to their less functional constraint than coding regions (Shaw *et al*., 2005). The *psbA-trnH* spacer has been proposed as the target DNA region for identifying Angiosperm species by using short orthologous DNA sequences (DNA barcodes) (Kress *et al*., 2005) while *petA-psbJ* spacer is a new cpDNA region used for studies of *Musa balbisiana* phylogeny (Swangpol, 2004) and phylogeography of *Trochodendron aralioides* (Huang *et al*., 2004). Therefore, these intergenic spacers in combination with *matK* were applied for examination of genetic diversity and phylogeny of Thai *Boesenbergia*.

The objectives of this study are to evaluate genetic variation and phylogenetic relationships of *Boesenbergia* in Thailand and to identify molecular markers that can be used for identifying species origin of these taxa. The basic information can then be practically applied to partially resolve taxonomic identification of *Boesenbergia* in Thailand.

Materials and Methods

Sampling. Twenty-two *Boesenbergia* taxa, representing 14 recognized species and 3 taxa (*Boesenbergia* sp.1-3) that could only be differentiated at the generic level were sampled (Table 1 and Fig. 1). These represent all four sections according to Loesener's (1930) classification. A representative of 4 closely related taxa (*Cornukaempferia aurantiflora*, *Hedychium biflorum*, *Kaempferia parviflora*, and *Scaphochlamys rubescens*) of *Boesenbergia* were included as outgroup references.

DNA extraction. Total DNA was extracted from fresh young leaves using a modification of the CTAB method of Doyle and Doyle (1987). After an addition of isopropanol, the precipitated DNA was gently hooked out with a pipette tip, placed in a new microcentrifuge tube, and washed with 70% ethanol. DNA was air-dried and resuspended in TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The concentration of extracted DNA was spectrophotometrically determined (Sambrook and Russell, 2001).

Polymerase chain reaction (PCR) and DNA sequencing. The *matK*, *psbA-trnH*, and *petA-psbJ*/*psbL* regions of each taxa were separately amplified in a 50-ml reaction volume containing 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.8% nonidet P40, appropriate $MgCl_2$ (4.0, 2.5 and 4.0 mM for *matK*, *psbA-trnH*, and *petA-psbJ*/*psbL*, respectively), dNTPs (0.28, 0.20 and 0.24 mM), primers (0.28 mM each of *matK*TI-1F; 5'-GTA GAG TCT ATA AGA CCA CGA-3' and *matK*TI-1R; 5'-CAC GAC TTT CCC TAT GTA TAC-3', 0.20 mM each of *psbA*-1F; 5'-CTT GGT ATG GAA GTA ATG CA-3' and *trnH*-1R; 5'-ATC CAC TTG GCT ACA TCC G-3', and 0.24 mM each of *petA*-F; 5'-AGG TTC AAT TGT MCG AAA TG-3' and *psbJ*-R; 5'-CTG GAA GRA TTC CTC TTT GG-3' or *psbL*-R; 5'-GTA CTT GCT GTT TTA TTT TC-3'; Fig. 2), total DNA (600-800, 200-400 and 200-400 ng, respectively) and 1 U of *Taq* DNA polymerase. PCR were carried out consisting an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (49, 50 or 58°C for 1 min), and extension (72°C for 3, 1 or 1 min). The final extension was carried out at 72°C for 10 min. PCR products were sequenced for both directions on an automated DNA sequencer using the original amplification primer as the sequencing primer except for sequencing of the 5' terminus of *matK* where TA-10F (5'-GAA GAT AGA TCT CGG CAA C-3'; Takano and Okada, 2002) and *matK*-R (5'-TTA CAT AAA AAT GTA TTC GCT C-3') were used.

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

Species	Geographic location	Remark
B. basispicata K. Larsen ex P. Sirirugsa	Nakhon Si Thammarat, PEN	2n = 20 (Augsonkitt *et al*., 2004)
B. curtisii (Bak.) Schltr.	Krabi, PEN	2n = 24 (Eksomtramage and Boontum, 1995)
B. curtisii (Bak.) Schltr.	Nakhon Si Thammarat, PEN	
B. longiflora (Wall.) Kuntze	Tak, N	
B. longiflora (Wall.) Kuntze	Phetchabun, NE	
B. longipes (Ridl.) Schltr.	Ranong, PEN	2n = 20 (Eksomtramage *et al*., 2002)
B. longipes (Ridl.) Schltr.	Nakhon Si Thammarat, PEN	
B. petiolata P. Sirirugsa	Saraburi, C	
B. plicata (Ridl.) Holttum	Krabi, PEN	2n = 20 (Eksomtramage *et al*., 1996), red flowers
B. plicata (Ridl.) Holttum	Krabi, PEN	2n = 20 (Eksomtramage *et al*., 1996 and Eksomtramage *et al*., 2002), yellow flowers
B. prainiana (Bak.) Schltr.	Narathiwat, PEN	2n = 20 (Eksomtramage *et al*., 1996)
B. pulcherrima (Wall.) Kuntze	Kanchanaburi, SW	
B. cf. *pulcherrima*1	Prachuap Khiri Khan, SW	
B. cf. *pulcherrima*2	Kanchanaburi, SW	
B. regalis B. Kharukanant & S. Tohdam	Narathiwat, PEN	
B. rotunda (L.) Mansf.	Not known	Thai common name 'Krachai Dang' (cultivated specimen)
B. rotunda (L.) Mansf.	Prachin Buri, SE	orange rhizomes
B. rotunda (L.) Mansf.	Chiang Mai, N	2n = 36 (Eksomtramage and Boontum, 1995), yellow rhizomes
B. siamensis (Gagnep.) P. Sirirugsa	Kanchanaburi, SW	
B. tenuispicata K. Larsen	Krabi, PEN	2n = 20 (Augsonkitt *et al*., 2004)
B. thorelii (Gagnep.) Loes.	Not known	cultivated specimen
B. xiphostachya (Gagnep.) Loes.	Mukdahan, NE	2n = 20 (Eksomtramage *et al*., 2002)
Boesenbergia sp.1	Nakhon Si Thammarat, PEN	
Boesenbergia sp.2	Uthai Thani, SW	variegated leaf
Boesenbergia sp.3	Tak, N	
Cornukaempferia aurantiflora J. Mood & K. Larsen	Phitsanulok, N	
Hedychium biflorum P. Sirirugsa & K. Larsen	Tak, N	
Kaempferia parviflora Wall.	Nakhon Ratchasima, E	
Scaphochlamys rubescens Jenjitt. & K. Larsen	Narathiwat, PEN	

N = Northern, NE = North-eastern, C = Central, E = Eastern, SE = South-eastern, SW = South-western, PEN = Peninsular Thailand.

PCR-restriction fragment length polymorphism (RFLP) of a partial *psbA-trnH* spacer. Nucleotide sequences of a *psbA-trnH* spacer of *Boesenbergia* and outgroups generated from *psbA*-1F and *trnH*-1R were aligned using BioEdit version 7.0.5.2 (Hall, 1999). A new reverse primer (*psbA*-3R; 5'-CAA GAA ACG ATA ATA AAT CTC G-3') was designed and tested against all investigated taxa. The amplification reaction was carried out in a 25- µl reaction volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM of each primer, 0.3 U of *Taq* DNA polymerase and 100 ng of DNA template. PCR was carried out using a touchdown profile involving predenaturation at 94°C for 3 min followed by 2 cycles of denaturation at 94°C for 45 s, annealing at 68°C for 1 min and extension at 72°C for 30 s, 9 cycles of the same profile with the exception that an annealing temperature was lowered for 2°C every 3 cycles and 28 cycles of the annealing temperature of 60°C. The final extension was performed at 72°C for 7 min. The PCR product was restricted with *Rsa* I (GTAC) at 37°C for 2.5 hrs. The digests were size-fractionated through 2.0% agarose gels prepared in 0.5X TBE, stained with ethidium bromide, and visualized under a UV transilluminator (Thaewnon-ngiw *et al*., 2004).

Phylogenetic analysis. Sequences were edited and aligned using BioEdit. The divergence between pairs of sequences was calculated using a Kimura (1980) 2-parameter model without indel consideration. For phylogenetic reconstruction, all three cpDNA sequence data were combined. Indels were accounted in the analysis (1 for insertion and 0 for deletion). Maximum parsimony analysis was carried out using a Phylogenetic Analysis Using Parsimony (PAUP*) software, version 4.0 Beta 10 (Swofford, 2004). The most parsimonious trees were obtained through the heuristic search option with 1000 random sequence addition. Bootstrapping (1000 replicates) was performed to assess levels of support for individual clades using the heuristic search with random sequence addition.

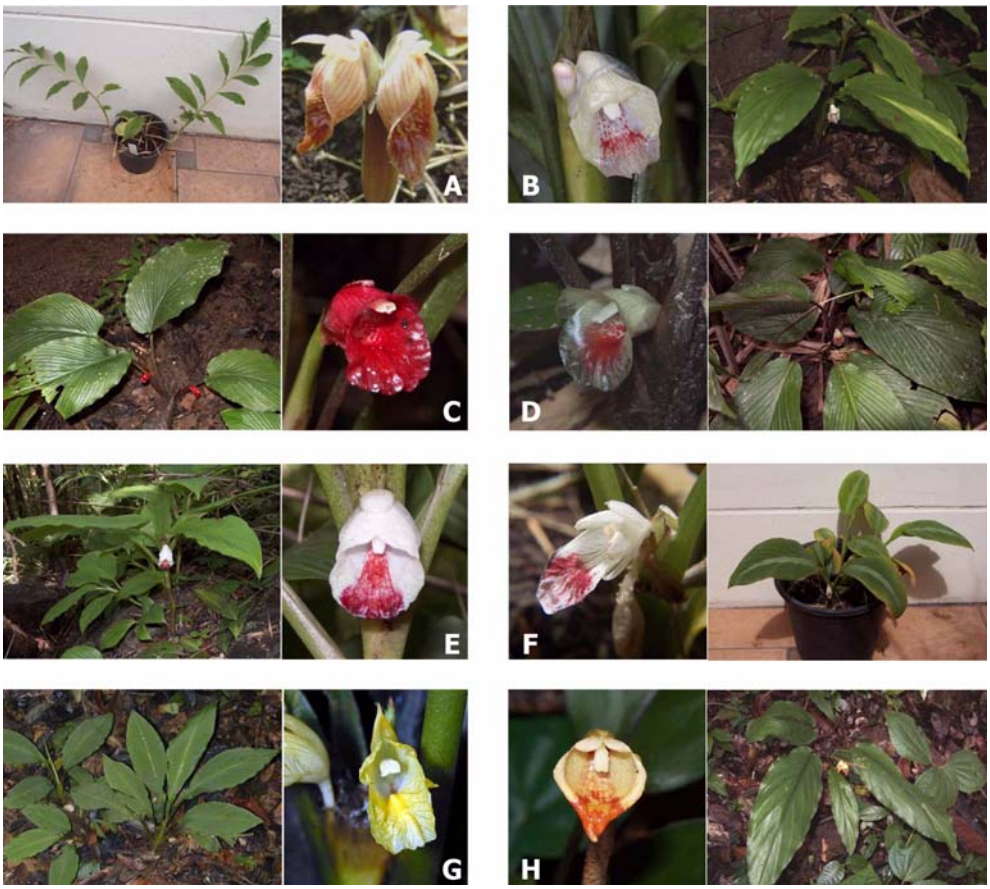

Fig. 1 External morphology of some *Boesenbergia* species in Thailand; *B. siamensis* (A), *B. petiolata* (B), *B. plicata* (red flowers, C), *B. plicata* (yellow flowers, D) *B. pulcherrima* (E), *Boesenbergia* sp.2 (variegated leaf, F), *B. regalis* (G) and *B. tenuispicata* (H).

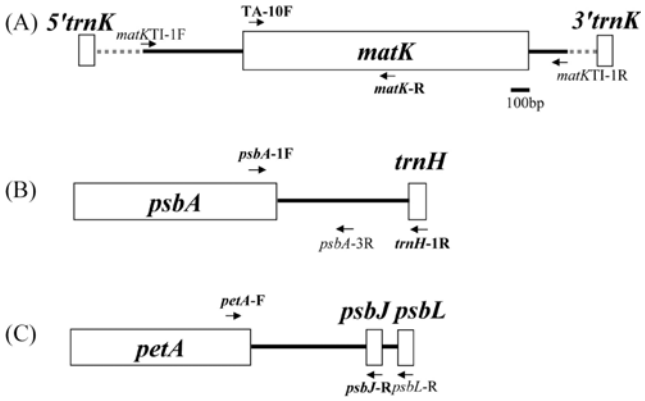

Fig. 2 Schematic diagrams illustrating positions of primers used for amplification and/or sequencing (boldface) of *matK* (A), *psbA-trnH* (B) and *petA-psbJ/psbL* (C) chloroplast DNA regions of *Boesenbergia* and outgroup references.

Results

The amplification products of *matK, psbA-trnH*, *petA-psbJ* and *petA-psbL* in Thai *Boesenbergia* were approximately 2.5 kb, 800 bp, 1.0 kb and 1.2 kb, respectively. The number of nucleotides after multiple alignments was 701 (*B. prainiana*) - 710 (*B. longiflora, B. longipes, B. petiolata, B. plicata, B. regalis* and *B. rotunda*) for *matK*, 714 bp (*B. curtisii*) - 750 bp (*B. longipes*) for *psbA-trnH* and 620 bp (*B. curtisii*) - 667 bp (*B. tenuispicata*) for *petA-psbJ* (Table 2). A 491 bp insertion was observed in a *psbA-trnH* spacer of *B. petiolata*.

Multiple alignments yielded the combined nucleotide sequences of 2803 bp in length. A total of 145 variable sites were found. These contained 45 phylogenetically informative characters, and 17 informative indels. Limited genetic diversity was observed from each chloroplast region (Table 2) resulting in low interspecific sequence divergence (0.25-1.74%) between *Boesenbergia* species. For species having replicate specimens, the intraspecific genetic variability was observed in *B. longiflora* (0.54%), *B. plicata* (0.05-0.10%), *B. pulcherrima* (0.00-0.15%) and *B. rotunda* (0.00-0.20%) but not in *B. curtisii* and *B. longipes*.

Polymorphic nucleotide sequences of *matK, psbA-trnH*, and *petA-psbJ* between *Boesenbergia* revealed species-specific orthologous markers in all investigated taxa (Table 3). Species origins of taxonomically unidentified *Boesenbergia* sp.1 and *Boesenbergia* sp.2, were confirmed to be *B. plicata* and *B. rotunda* while *B. pulcherrima* (Mahidol University, Kanchanaburi)

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

	matK	*psbA-trnH*[a]	*petA-psbJ*	combined data
Number of nucleotides	701 – 710	714 – 1252	620 - 667	2803
Number of variable sites	45	49	51	145
Number of informative characters	16	19	10	45
Number of informative indels	3	7	7	17
Percentage of interspecific and intergeneric sequence divergence*	0.15 - 1.72	0.00 - 3.53	0.00 – 2.53	0.25 – 1.74
Percentage of intraspecific sequence divergence				
B. curtisii	0.00	0.00	0.00	0.00
B. longiflora	0.44	0.83	0.31	0.54
B. longipes	0.00	0.00	0.00	0.00
B. plicata	0.00	0.00-0.14	0.00-0.16	0.05-0.10
B. pulcherrima	0.00-0.45	0.00	0.00	0.00-0.15
B. rotunda	0.00	0.00-0.14	0.00-0.65	0.00-0.20

[a]An insertion of 491 bp was found in *B. petiolata*, *indels were not included in the analysis

and *B.* cf. *pulcherrima*1 (Prachuap Khiri Khan) and *B.* cf. *pulcherrima*2 (Thong Pha Phum, Kanchanaburi) exhibiting floral variation (Fig. 3) were all recognized as *B. pulcherrima*. In addition, morphologically similar *Boesenbergia* sp.3 and *B. longiflora* displayed different species-diagnostic sequences.

Phylogenetic analysis based on the combined sequences of *matK, psbA-trnH* and *petA-psbJ* generated 96 most parsimonious trees with the equal length of 208 steps and consistency (CI) and retention (RI) indices of 0.72 and 0.83, respectively. A 50% majority-rule consensus tree divided *Boesenbergia* into 3 groups including: A (*B. longiflora*, *B. basispicata*, *B. tenuispicata*, *B. prainiana*, *B. plicata*, *B. longipes*, *B. xiphostachya*, *B. petiolata*, *B. pulcherrima*, *B. thorelii*, *Boesenbergia* sp.1 and *Boesenbergia* sp.3), B (*B. curtisii*, *B. rotunda*, *B. regalis* and *Boesenbergia* sp.2) and C (*B. siamensis*). Identical *Boesenbergia* species originating from different geographic locations were phylogenetically clustered together with significant bootstrapping values (Fig. 4).

Considering nucleotide sequences of a partial *psbA-trnH* spacer, an insert of CTT(A/C/T)GTGTA was found in all *Boesenbergia* allocated into the clade A but not B and C. Additionally, the 5' adjacent region of an insertion possesses an *Rsa* I recognition site allowing the development of PCR-RFLP to differentiate *Boesenbergia* lineages (the presence of restriction site; clade A and the absence of restriction site; clades B and C) derived from phylogenetic analysis of the chloroplast DNA sequences (Fig. 5).

Discussion

Boesenbergia is one of the systematically problematic genera in Zingiberaceae. Taxonomy of these species relied based primarily on external morphology. Authentication of *Boesenbergia* is rather difficult due to heterogeneity of external morphology at both intraspecific and interspecific levels. In the present study, the reliable molecular taxonomic key of *Boesenbergia* in Thailand was successfully developed based on polymorphism of *matK*, *psbA-trnH* and *petA-psbJ*.

Limited sequence divergence of the partial *matK* was observed at both interspecific and intergeneric levels (0.15-1.72%, 16 informative sites and 3 indels). Apparently, informative characters and indels of *matK* in *Boesenbergia* are lower than those in *Globba* (37 informative sites and 9 indels; Takano and Okada, 2002) and other genera. This suggested that polymorphism of *matK* may not be sufficient to differentiate all investigated taxa in this study. Subsequently, *psbA-trnH* and *petA-psbJ* intergenic spacers were included and revealed slightly greater divergence between investigated taxa (0.00-3.53% and 0.00-2.53% with additional 26 and 17 informative sites/indels, respectively).

Kress *et al.* (2005) proposed ITS and *trnH-psbA* intergenic spacer to be potentially usable DNA regions for species identification using polymorphic DNA sequences (DNA barcodes) to facilitate biodiversiy studies of 99 plant species belonging to 80 genera of 53 families.

Likewise, nucleotide sequences of the combined chloroplast DNA revealed informative sites and indels that can be used for species diagnosis of Thai *Boesenbergia* in this study unambiguously and is reported for the first time here. Several taxa, for instance *Boesenbergia* sp.1 and *Boesenbergia* sp.2, were able to morphologically identify only at the generic level. Using species-diagnostic sequences found in this study, these taxa were recognized as an ecomorphological variant of *B. plicata* and *B. rotunda*, respectively. Additionally, *B.* cf. *pulcherrima* inferred from leafy shoots and storage roots were collected without the reproductive parts from Prachuap Khiri Khan (peninsular Thailand) and Kanchanaburi (south-western

Table 3. Polymorphic SNP and indels for identifying species origins of Thai *Boesenbergia*

Species	Nucleotide position																							
	mat K*								***psbA-trnH*****					***petA-psbJ*****										
	136	144	388	456	480	516	605	629	733	790	865	1870	2047	2050	2083	2163	2182-2186	2241	2305	2598	2644	2712-2716	2753	2776-2784
Boesenbergia sp.3[a]	C	C	C	G	C	C	C	A	C	A	T	A	G	G	C	C	TTGAT	G	A	A	C	TTCCA	G	TCTTATAT
B. basispicata	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. curtisii Krabi	.	.	.	.	.	.	.	.	.	-	.	G	T	.	.	.	-	.	.	.	.	TTCCA	.	-
B. curtisii Nakhon Si Thammarat	.	.	.	.	.	.	.	.	.	-	.	G	T	.	.	.	-	.	.	.	.	TTCCA	.	-
B. longiflora Tak[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TTGAT	.	.	.	.	TTCCA	.	TCTTATAT
B. longiflora Phetchabun[a]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TTGAT	.	.	.	.	TTCCA	.	TCTTATAT
B. longipes Ranong	.	T	.	.	.	T	.	C	.	C	C	G	.	.	.	.	-	.	.	G	T	-	A	TCTTATAT
B. longipes Nakhon Si Thammarat	.	T	.	.	.	T	.	C	.	C	C	G	.	.	.	.	-	.	.	G	T	-	A	TCTTATAT
B. petiolata	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. plicata (red flower)	A	.	.	.	T	.	T	.	T	C	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. plicata (yellow flower)	A	.	.	.	T	.	T	.	T	C	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
Boesenbergia sp.1	A	.	.	.	T	.	T	.	T	C	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. prainiana	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	-	.	T	.	.	TTCCA	.	TCTTATAT
B. pulcherrima	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. cf. *pulcherrima* 1	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. cf. *pulcherrima* 2	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. regalis	.	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
Boesenbergia sp.2 (variegated leaf)	.	.	T	A	.	.	.	.	.	-	.	G	T	.	A	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. rotunda 'Krachai Dang'	.	.	T	A	.	.	.	.	.	-	.	G	T	.	A	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. rotunda (orange rhizome)	.	.	T	A	.	.	.	.	.	-	.	G	T	.	A	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. rotunda (yellow rhizome)	.	.	T	A	.	.	.	.	.	-	.	G	T	?	?	?	?	?	?	?	?	?	?	?
B. siamensis	.	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	-	T	.	.	.	TTCCA	.	TCTTATAT
B. tenuispicata	.	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. thorelii	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
B. xiphostachya	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	T	-	.	.	.	.	TTCCA	.	TCTTATAT
C. aurantiflora	.	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	-	.	C	.	.	TTCCA	.	TCTTATAT
H. biflorum	.	.	.	T	.	.	.	.	.	-	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
K. parviflora	.	.	.	.	.	.	.	.	.	-	.	G	T	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT
S. rubescens	.	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	-	.	.	.	.	TTCCA	.	TCTTATAT

? = direct sequencing of *petA-psbJ* of *B. rotunda* (yellow rhizomes) was unsuccessful. * = GenBank accession no. DQ408336-408364, ** = GenBank accession no. DQ408307-408335, *** = GenBank accession no. DQ104857-104879 and DQ409818.

[a]Further differentiation of these taxa can be carried out by the presence of a 25 bp insertion of CTTGTATCCTATTCTTTGTAATAGA (position 2095-2119) in *petA-psbJ* apacer of *Boesenbergia* sp.3.

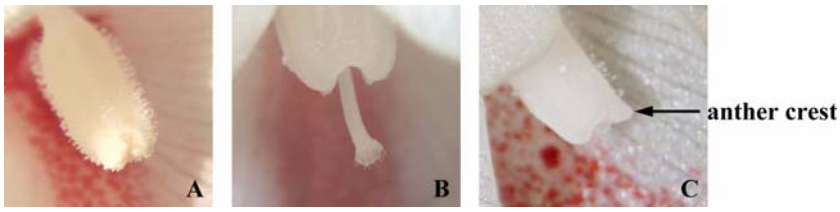

Fig. 3 Morphological variation of *B. pulcherrima* originating from Mahidol University, Kanchanaburi (A) and Prachuap Khiri Khan (B) that lack the anther crests and that originating from Thong Pha Phum, Kanchanaburi (C) that possesses the anther crests. They are regarded as *B. pulcherrima* based on species-diagnostic sequences of *matK*, *psbA-trnH*, and *petA-psbJ* (see text for detailed information).

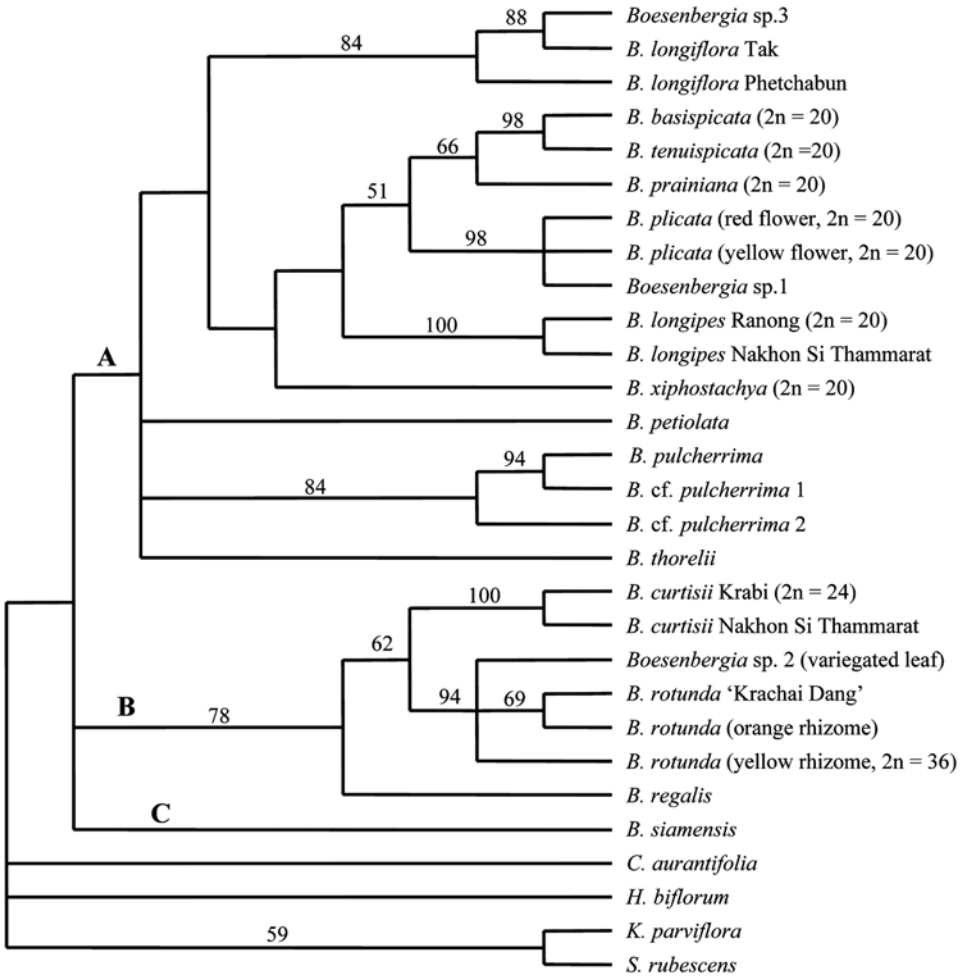

Fig. 4 A 50% majority-rule consensus tree constructed from the 96 most parsimonious trees based on combined *matK*, *psbA-trnH*, and *petA-psbJ* sequences (tree length = 208 steps; consistency index, CI = 0.72 and retention index, RI = 0.83). Values at the node (>50%) represent the percentage of times that the particular node occurred in 1000 replicates generated by bootstrapping the original sequences. Differentiation between lineages were compatible with the chromosome numbers (2n = 20 for members of clade A and 2n = 24 and 36 for those of clade B).

Thailand). They are *B. pulcherrima* according to species-specific sequences of the chloroplast DNA. Owing to our finding, SNP-based PCR assays can be further developed to simplify the detection method from DNA sequencing to allele-specific polymerase chain reaction (Moorhead *et al*., 2003 and Miles *et al*., 2004).

The external characteristics of the anther crests have been used for classification of *Boesenbergia*. For example, *B. pulcherrima* has crest bilobed, shortly extended beyond thecae (Sirirugsa, 1992). The vegetative parts of this presumptive species were collected from 3 different geographic locations and cultivated. Two of which lacked the anther crests (Fig. 3).

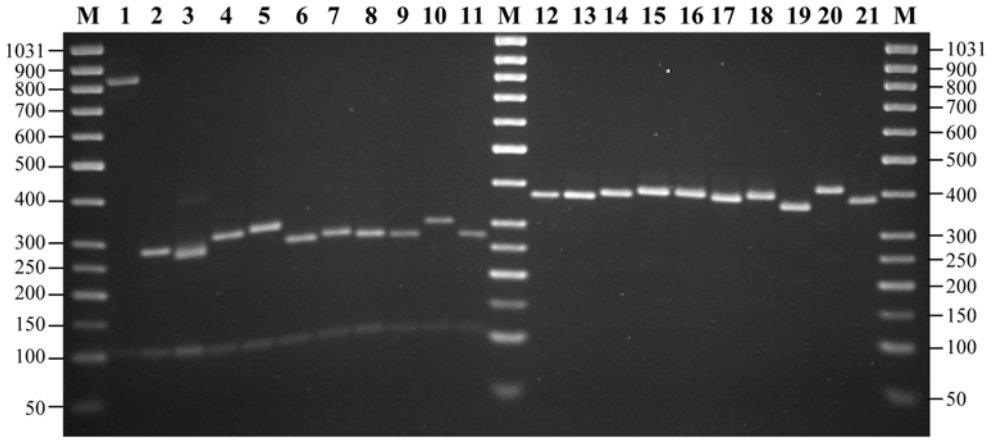

Fig. 5 Agarose gel electrophoresis illustrated restriction analysis of *Rsa*I-digested partial *psbA-trnH* spacer of *B. petiolata* (lane 1), *Boesenbergia* sp.3 (lane 2), *B. basispicata* (lane 3), *B. longiflora* (lane 4), *B. longipes* (lane 5), *B. plicata* (lane 6), *B. prainiana* (lane 7), *B. pulcherrima* (lane 8), *B. tenuispicata* (lane 9), *B. thorelii* (lane 10), *B. xiphostachya* (lane 11), *B. curtisii* (Krabi, lane 12), *B. curtisii* (Nakhon Si Thanmmarat, lane 13), *B. regalis* (lane 14), *Boesenbergia* sp.2 (variegated leaf, lane 15), *B. rotunda* ('Krachai Dang', lane 16), *B. siamensis* (lane 17), *C. aurantiflora* (lane 18), *H. biflorum* (lane 19), *K. parviflora* (lane 20) and *S. rubescens* (lane 21). A 50 bp ladder (lanes M) was included as the DNA marker.

Floral morphology and species-diagnostic sequences of these plants suggested that they are *B. pulcherrima*. This illustrated that the anther crests cannot be used for identification of *B. pulcherrima*.

According to external morphology, *Boesenbergia* sp.3 was *B. longiflora*-like. Nevertheless, flowers of *Boesenbergia* sp.3 are straw-colored, with the strongly crinkled labellum margin whereas those of *B. longiflora* are white to pink, with the faintly undulated labellum margin (K. Larsen, personal communication). Species-specific nucleotide sequences deduced from *matK*, *psbA-trnH* and *petA-psbJ* polymorphism indicating that *Boesenbergia* sp.3 is not *B. longiflora* but it should have been a newly unidentified *Boesenbergia* species in Thailand. In contrast, informative sequences of *Boesenbergia* species exhibiting intraspecific morphological variation (e.g. in *B. rotunda* or *B. plicata* exhibiting different rhizome or flower colors) were perfectly identical confirming the species status of allopatric taxa.

Vanijajiva *et al*. (2003 and 2005) examined phylogenetic relationships between 11 *Boesenbergia* taxa representing 8 extant species using isozyme (POX, SOD, GDH and MDH) and RAPD (OPB-14, OPAM-01, OPAM-03, OPAM-12, and OPZ-03) analyses. UPGMA dendrograms constructed from genetic similarity in their studies were comparably separated overall investigated taxa to 2 different groups; A (*B. basispicata*, *B. prainiana*, *B. pulcherrima* and *B. tenuispicata*) and B (*B. rotunda* and *B. curtisii*). Nevertheless, *B. longipes* (RAPD) and *B. plicata* (isozymes and RAPD) were allocated in the same group of *B. rotunda* (2n = 36) and *B. curtisii* (2n = 24) while results from *matK*, *psbA-trnH*, and *petA-psbJ* sequences clearly allocated these taxa which possess 2n = 20, to a different phylogenetic lineage.

Notably, the inheritance of cpDNA is usually uniparental and effectively haploid. Therefore, studies of cpDNA polymorphism cannot be used to examine genetic contribution by the other parental lineage. However, the effective population size estimated from cpDNA is generally smaller than that estimated from nuclear markers such as allozymes and nuclear DNA (Birky *et al*., 1989). This increases the effects of genetic drift and the rate of turnover within populations which provides more rapid sorting of ancestral alleles within and between species than nuclear DNA markers (Dowling, 1996). Moreover, slightly differences of the detail grouping between *Boesenbergia* previously reported by Vanijajiva *et al*. 2003 and 2005 and this study may have resulted from the use of molecular markers exhibiting different evolutionary rates (e.g. between protein and DNA markers and between nuclear and organelle DNA) and/or different approaches (isozyme electrophoresis, RAPD-PCR and DNA sequencing).

Interestingly, phylogenetic analysis based on *matK*, *psbA-trnH* and *petA-psbJ* polymorphism allocated *Boesenbergia* species to 3 separate groups which are concordant with inflorescence types; A (elongated inflorescences), B (compact inflorescences) and C (*B. siamensis* in which the inflorescence appears before leafy shoots after the dormancy period). The somatic chromosome numbers of previously characterized *Boesenbergia* species possessed the chromosome number of 2n = 20 were allocated into the clade A whereas *B. curtisii* and *B. rotunda* possessed 2n = 24 and 2n = 36 were allocated to the clade B, respectively (Eksomtramage and Boontum, 1995; Eksomtramage *et al*., 1996; Eksomtramage *et al*., 2002 and Augsonkitt *et al*., 2004). This suggested the existence of evolutionarily separated lineages in *Boesenbergia* as previously reported based on the combined ITS and *matK* region sequence data (Kress *et al*., 2002) and ITS and *trnL-trnF* (Ngamriabsakul *et al*., 2003). Therefore, it is interesting to examine whether the chromosome numbers of *Boesenbergia*

sp. 1, 2 and 3 and the remaining uninvestigated Thai *Boesenbergia* reflected evolutionary lineages found in this study.

Kress *et al.* (2002) studied phylogenetic relationships of 3 species of *Boesenbergia* (*B. rotunda*#1 and #2, *B. pulcherrima* and *B. longiflora*#1 and #2) and all genera of the Zingiberaceae and found that *Boesenbergia* were paraphyletic when genetically analyzed by ITS but possibly monophyletic by *matK* sequence data. Although the monophyly of *Boesenbergia* cannot be concluded by our results (only other four related genera were included in the analysis), all investigated *Boesenbergia* taxa clustered together. The use of all related genera and an outgroup outside Zingiberaceae would clarify the phylogenetic controversy of *Boesenbergia*.

Our present study illustrated the use of *matK*, *psbA-trnH* and *petA-psbJ* polymorphism to examine intra- and interspecific variability, phylogenetic relationships and species-diagnostic markers to assist taxonomic identification of Thai *Boesenbergia*. Lineage-specific detection by PCR-RFLP and species-specific sequences found in this study can be directly applied for authentication of fresh or dried plant materials to ensure reliable botanical sources of metabolic profiling studies of these species.

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