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Confirmation of relationships among *Boesenbergia* (Zingiberaceae) and related genera by RAPD

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Abstract

The relationships among 19 accessions of Zingiberaceae belonging to 11 species of *Boesenbergia*, six species of *Kaempferia*, and two species of *Scaphochlamys* from Southern Thailand were studied using random amplified polymorphic DNA (RAPD) profiles from leaf tissue samples. The RAPD was carried out using 10 random decamer arbitrary primers. Amplification occurred in five out of 10 tested primers (OPAM-01, OPAM-03, OPAM-12, OPB-14, OPZ-03). Total of 53 amplified bands were observed. Data obtained from the RAPD fingerprints from the samples clarified some doubts in morphological classification. The data were analyzed for the Nei and Li's Dice similarity coefficient for pair-wise comparison between individual samples and the distance matrix. The dendrogram resulting from cluster analysis, UPGMA and a principal component analysis of the RAPD result confirms a higher degree of relationship between *Boesenbergia* and *Scaphochlamys* than between *Boesenbergia* and *Kaempferia*.

Keywords: RAPD; *Boesenbergia*; Zingiberaceae; Genetic relationship

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1. Introduction

Boesenbergia Kuntz, *Kaempferia* L., and *Scaphochlamys* Baker. are genera of Zingiberaceae distributed in Southeast Asia (Larsen et al., 1998). They are classified in the tribe Hedychieae (Holttum, 1950) but recently Kress et al. (2002) suggested that they should be treated as subfamily Zingiberoideae, tribe Zingibereae. The three genera were regarded as closely related by Holttum (1950). They are small herbaceous plants with short, fleshy or slender rhizomes, one to a few leaves, similar appearance in vegetative characters and occurring in similar habitats. Several species are cultivated for food, spice and folk medicine. An extract of rhizomes exhibits anti-inflammatory and HIV-1 protease inhibitory activity (Tuchinda et al., 2002; Tewtrakul et al., 2003). Breeding is seriously handicapped by poor fruit setting (Larsen et al., 1999). There are many variations in color, for instance, *B. curtisii* can have black or white leaf-sheaths and *B. plicata* can have yellow or red flowers. Kress et al. (2002) also found that defining characters are not universal for all taxa within tribes of the family Zingiberaceae. Therefore, a more accurate method for distinguishing species within these three genera is needed. They used internal transcribed spacer (ITS) and plastid *matK* from 104 species in the family to make a new classification of the family Zingiberaceae and showed that *Boesenbergia* is polyphylectic. *Boesenbergia* is believed to be closer to *Scaphochlamys* than to *Kaempferia* as judged by their morphology (Hussin et al., 2001), and we successfully supported this by using isozyme electrophoresis (Vanijajiva et al., 2003). However, the isozyme patterns may be affected by different environments and the stage of plant development. Moreover, only limited number of isozymes loci are available for certain taxa (Mangolin et al., 1997; Garkava et al., 2000).

Random amplified polymorphic DNA (RAPD) markers were introduced in 1990 (Williams et al., 1990). Their use as molecular markers for taxonomic and systematic analyses of plants (Bartish et al., 2000), as well as in their breeding and genetic relationships has increased exponentially (Ranade et al., 2001), due to easiness of the procedure. Apparently, RAPD markers evolve more quickly than the isozyme method. RAPD uses small amounts of the sample for analysis and detects variation in both coding and non-coding regions of the genome while morphological and isozyme variations only reflect difference in protein-coding sequences (Adam, 1999; Sharma and Jana, 2002). RAPD has been used to distinguish or assist identifying some plants as well as several species in Zingiberaceae such as *Zingiber officinales* (Hsiang and Huang, 2000; Rout et al., 1998), *Curcuma aeruginosa* (Prathepha, 2000), *Alpinia* spp., *Curcuma* spp., *Etlingera* spp., *Zingiber* spp. (Dasuki et al., 2000) *Curcuma* spp. (Wangsomnuk et al., 2002).

The aim of this study is to confirm the phenotypic identification, and the relationships previously reported on the taxa of *Boesenbergia*, *Kaempferia* and *Scaphochlamys* (Hussin et al., 2001; Kress et al., 2002; Vanijajiva et al., 2003), by using RAPD. Variation in morphology of *B. curtisii* and *B. plicata* is also checked whether this is a reflection of difference at the genetic level at both transcribing and non-transcribing regions. The RAPD result will exclude the effect of environments and the stage of plant development.

2. Materials and methods

2.1. Plant materials

DNA isolation and RAPD analysis were carried out on samples from 19 taxa collected from seven provinces in Southern Thailand (Fig. 1). These samples include 11 taxa of *Boesenbergia*, six taxa of *Kaempferia*, and two taxa of *Scaphochlamys* (Table 1). Most of the samples are representative of the species showing morphological characteristics, except *B. curtisii* (B2), (B3) and *B. plicata* (B5), (B6) showing little phenotypic variability. All species were cultivated in a greenhouse at the Faculty of Science, Prince of Songkla University, Hat Yai Campus. Voucher specimens of all accessions are deposited in Prince of Songkla University Herbarium.

2.2. Genomic DNA isolation

Genomic DNA was extracted from the leaves of 19 accessions, using the CTAB method following the procedure of Doyle and Doyle (1987) with minor modifications. The leaves (0.05 g) were ground in a mortar with a pestle. Extraction buffer [(1% (w/v) CTAB, 50 mM Tris–HCl (pH 8), 0.7 M NaCl, 0.1% β-mercaptoethanol)] 500 μl was added and the solution was incubated at 60 °C for 30 min. The homogenate was mixed with 25:24:1 phenol:chloroform:isoamyl alcohol (v/v/v) by gentle inversion. After centrifugation at 13 000 rpm for 15 min, the upper aqueous layer was transferred to a fresh tube. RNA was removed by treating with 2.5 μl of the RNase (10 mg/ml) for 30 min at 37 °C. The extraction of DNA with phenol/chloroform/isoamyl alcohol was repeated one more time. DNA in the solution was precipitated with 0.6 volume of ice-cold isopropanol and washed with 70% ethanol. Following this, the DNA was extracted using CTAB DNA extraction protocol without RNase. The process was repeated until the DNA pellet was free of color (two to three times) and the final pellet was dissolved in sterile deionized water. DNA quality and quantity were determined on 0.8% agarose gel. The DNA was stored at −20 °C, for further use as templates for PCR amplification.

2.3. RAPD-PCR analysis

To optimize the PCR amplification condition, experiments were carried out with varying concentrations of $MgCl_2$ and DNA template. Six different concentrations of $MgCl_2$ (2.5, 3, 3.5, 4, 4.5, 5 mM) and three different concentrations of DNA template (25, 50, 100 ng) were used.

PCR mixture (25 μl) contained: 10× Promega reaction buffer (100 mM Tris–HCl pH 9, 500 mM KCl, 1% Triton X-100), 0.4 mM of each dNTP, 0.6 μM of primer, 0.5 unit of Taq polymerase (Promega), 5 mM $MgCl_2$ and 100 ng template DNA. Each reaction was overlaid with 10 μl of mineral oil to prevent evaporation. Ten decanucleotide primers (Operon Technologies, California, USA) of random sequences were used (Table 2).

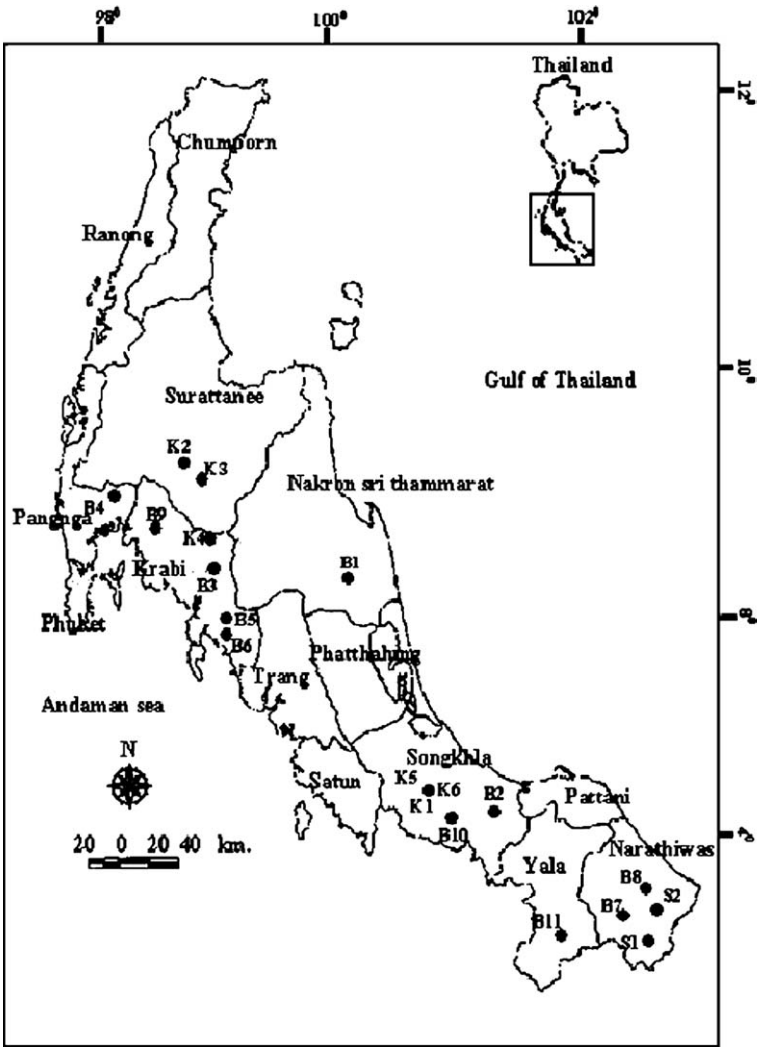

Fig. 1. Southern Thailand with location where plants were collected.

PCR was performed using a Hybaid thermocycler, programmed for an initial melting step at 94 °C for 4 min, followed by 45 cycles, each cycle consisting of three steps of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. A final extension step at 72 °C for 4 min was performed after the 45 cycles. A negative control reaction in which DNA was omitted was included in every run in order to verify the absence of contamination. The RAPD products were separated by agarose (1.8% w/v) gel electrophoresis at 75 V for 2.5 h in 0.04 M TAE (Tris–acetate 0.001 M-EDTA) buffer pH 8. The gels were stained with ethidium bromide (10 mg/ml), and photographed on a UV transluminator. To determine RAPD profiles, the size of

Table 1
Samples of *Boesenbergia*, *Kaempferia* and *Scaphochlamys* used in this study

Taxa	Vouchers	Sample number
Boesenbergia		
B. basispicata Larsen ex Sirirugsa	OVT001	(B1)
B. curtisii (Bak.) Schltr. with black leaf-sheath	OVT002	(B2)
B. curtisii (Bak.) Schltr. with white leaf-sheath	OVT003	(B3)
B. longipes (King & Prain) Schltr.	OVT004	(B4)
B. plicata (Ridl.) Holtt. with red flower	OVT005	(B5)
B. plicata (Ridl.) Holtt. with yellow flower	OVT006	(B6)
B. prainiana (King ex Bak.) Schltr.	CMT001	(B7)
B. prainiana (King ex Bak.) Schltr.	CMT001	(B7)
B. pulcherrima (Wall.) Kuntz	CMT002	(B8)
B. tenuispicata K. Larsen	OVT007	(B9)
B. rotunda (L.) Mansf.	OVT008	(B10)
B. aff. *rotunda* (L.) Mansf.	CMT003	(B11)
Kaempferia		
K. angustifolia Rosc.	OVT009	(K1)
K. elegans Wall.	OVT010	(K2)
K. galanga L.	OVT011	(K3)
K. pulchra Ridl.	OVT012	(K4)
K. siamensis P. Sirirugsa	OVT013	(K5)
K. roscoeana Wall.	OVT014	(K6)
Scaphochlamys		
S. biloba (Ridl.) Holtt.	CMT004	(S1)
S. perakensis Holtt.	CMT005	(S2)

each DNA band was inferred by comparison with a 1 kb ladder (Promega), used as a molecular weight marker (M). Polymorphisms at all loci were confirmed by three repeating tests for each primer at different times.

2.4. Gel scoring and data analysis

Only strong and reproducible RAPD bands were scored. Different patterns observed were scored as discrete variables, using 1 to indicate the presence and 0 to indicate the absence of a unique pattern. The SPSS (version 9.01) data analysis package (Norusis, 1990) was used for the statistical analyses (Backeljau et al., 1996). Relationships among individuals were determined by the distance matrix method. Nei and Li's Dice similarity coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix (Nei and Li, 1979). A dendrogram was constructed from this matrix on the basis of the hierarchical cluster analysis, which is based on the average linkage between group, i.e. the unweighed pair-group method algorithm (UPGMA) as described by Sneath and Sokal (1973). A principal component analysis (PCA) was also conducted using a genetic distance matrix obtained from the binary data set. It was negated and rescaled (0–1), using the Euclidean distance between pair-wise comparison of individuals (Ludwig and Reynolds, 1988).

Table 2
Nucleotide sequence of the 10 decanucleotide primers used in this study

Primer number	Nucleotide sequence 5′ to 3′	% GC
OPAM-01	TCACGTACGG	60
OPAM-03	CTTCCCTGTG	60
OPAM-12	TCTCACCGTC	60
OPAM-18	ACGGGACTCT	60
OPB-01	GTTTCGCTCC	60
OPB-14	TCCGCTCTGG	70
OPC-01	TTCGAGCCAT	60
OPC-05	GATGACCGCC	70
OPK-05	TCTGTCGAGG	60
OPZ-03	CAGCACCGCA	70

3. Results and discussion

DNA extracted from *Boesenbergia*, *Kaempferia* and *Scaphochlamys* using a modified Doyle and Doyle (1987) method gave red or brown-colored DNA. The DNA did not give any band for visualization after RAPD-PCR, possibly because the presence of the phenolic compound that intercalates irreversibly with the DNA helices as reported in the case of DNA from another group of tropical plants (Rajaseger et al., 1997; Rath et al., 1998). The extraction of high quality DNA was optimized by re-extracting the DNA using CTAB DNA extraction protocol and phenol:chloroform:isoamyl alcohol extraction instead of chloroform: isoamyl alcohol extraction. The phenolic compound and pigment co-precipitating with the DNA were easily removed and good RAPD profiles were obtained with all samples.

A necessary precondition for RAPD analysis is the establishment of PCR conditions that ensure reliable and reproducible results (Ramser et al., 1996). In our PCR reactions, the concentrations of 100 ng of DNA template and 5 mM $MgCl_2$ gave the best result. Among the 10 random primers used for initial screening, five primers (OPAM-01, OPAM-03, OPAM-12, OPB-14 and OPZ-03) gave optimum RAPD profiles with all the taxa studied. Fifty-three bands were generated using the five primers. The amplified product varied between 100 and 2000 bp. Representative RAPD profiles generated with OPAM-03 and OPZ-03 for the taxa studied are shown in Fig. 2. Variations in color of leaf-sheaths and flowers, occur in *Boesenbergia*, for example, *B. curtisii* B2 and B3 or *B. plicata* B5 and B6 can be detected by RAPD markers. The results showed that morphological variations within the species resulted from differences at genetic level, similar result has also been reported by Kress et al. (2002). They found that *B. rotunda* 1 from Hawaii and *B. rotunda* 2 from Mynmar also showed different results from ITS and *matK* (Kress et al., 2002). The genetic similarity among 19 taxa examined using RAPD patterns and Dice similarity coefficients for pair-wise comparison between individuals is shown in Table 3. The similarity coefficient ranges from 0.367 to 0.97, among the 11 species of *Boesenbergia* it runs from 0.54 to 0.853, with the lowest value obtained for

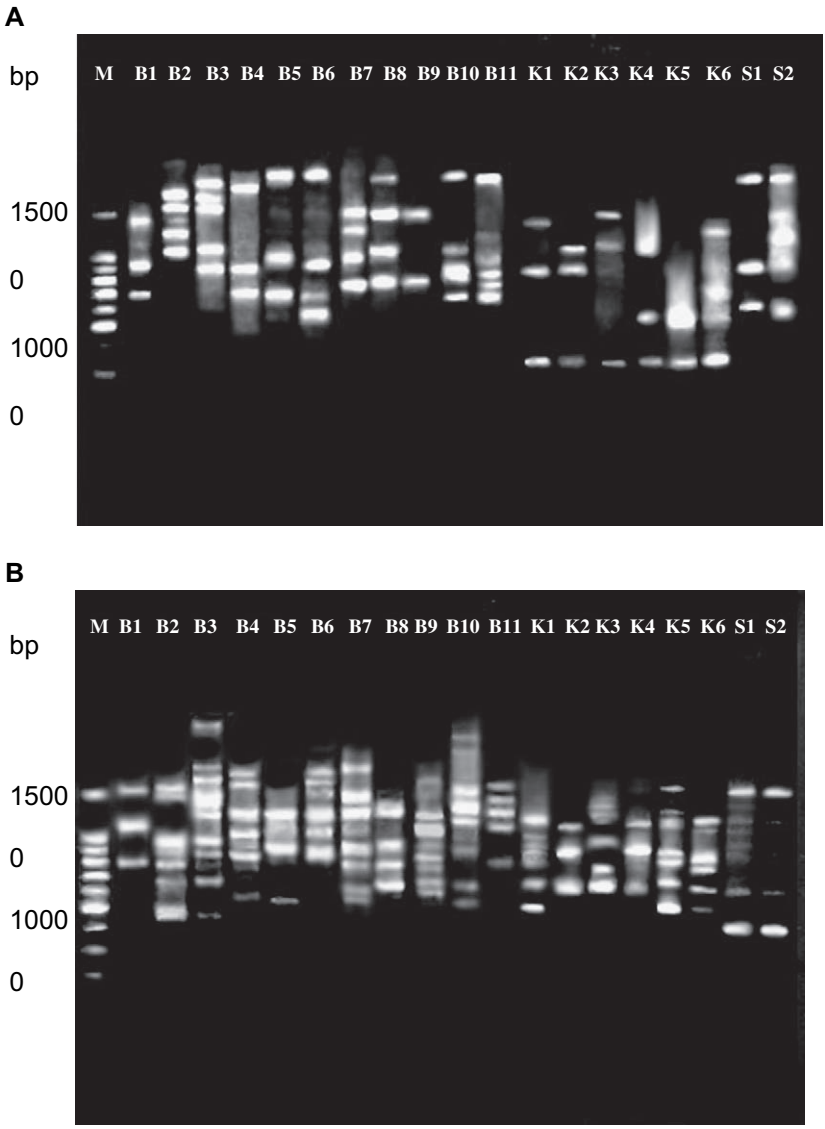

Fig. 2. Examples of the RAPD polymorphisms from 19 taxa revealed by decanucleotide primers (A) OPAM-03 and (B) OPZ-03 (left to right: lane M, molecular weight marker = 1 kb ladder DNA; lanes B1–S2 stand for individual species of plants, for detail see in Plant materials).

B1–B3, whereas B10 and B11 show the highest similarity values within the genus. In *Kaempferia*, a larger similarity is obtained (0.59–0.97), the lowest being between K1 and K4 whereas K2 and K4 show the highest similarity value. The two species of *Scaphochlamys* show similarity value 0.909. The intergeneric similarity ranges from 0.367 to 0.615 for *Boesenbergia* and *Kaempferia*, from 0.383 to 0.615 for

Table 3
Pair-wise genetic similarity of 19 taxa according to the index of Nei and Li (1979)

Taxa	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	K1	K2	K3	K4	K5	K6	S1	S2
B1	1.000																		
B2	0.576	1.000																	
B3	0.540	0.853	1.000																
B4	0.654	0.737	0.689	1.000															
B5	0.667	0.677	0.725	0.621	1.000														
B6	0.610	0.656	0.735	0.632	0.862	1.000													
B7	0.755	0.621	0.581	0.706	0.712	0.690	1.000												
B8	0.642	0.621	0.581	0.549	0.746	0.690	0.769	1.000											
B9	0.778	0.678	0.571	0.615	0.667	0.644	0.755	0.717	1.000										
B10	0.702	0.613	0.697	0.582	0.698	0.677	0.643	0.679	0.667	1.000									
B11	0.633	0.708	0.754	0.724	0.818	0.738	0.644	0.644	0.700	0.825	1.000								
K1	0.511	0.577	0.500	0.533	0.528	0.538	0.565	0.565	0.553	0.560	0.566	1.000							
K2	0.512	0.375	0.462	0.390	0.490	0.458	0.476	0.476	0.558	0.522	0.531	0.611	1.000						
K3	0.571	0.593	0.552	0.553	0.582	0.593	0.583	0.583	0.531	0.615	0.582	0.952	0.632	1.000					
K4	0.500	0.367	0.453	0.381	0.480	0.449	0.465	0.465	0.545	0.511	0.520	0.595	0.970	0.615	1.000				
K5	0.549	0.536	0.533	0.449	0.561	0.571	0.520	0.560	0.549	0.556	0.526	0.864	0.650	0.870	0.683	1.000			
K6	0.489	0.400	0.444	0.372	0.510	0.520	0.455	0.409	0.489	0.500	0.471	0.632	0.824	0.700	0.857	0.762	1.000		
S1	0.449	0.407	0.517	0.383	0.582	0.556	0.458	0.542	0.408	0.615	0.582	0.429	0.579	0.500	0.564	0.565	0.550	1.000	
S2	0.490	0.444	0.552	0.426	0.582	0.556	0.500	0.542	0.408	0.577	0.545	0.400	0.474	0.500	0.462	0.565	0.450	0.909	1.000

Boesenbergia and *Scaphochlamys* and from 0.400 to 0.579 for *Kaempferia* and *Scaphochlamys* (Table 3).

Cluster analysis and principal component analyses are valuable for determining relationships among populations of the same and different species (Crawford, 1990). The phenetic analysis of all five primers gave much more complete information than if each primer was analyzed separately. Pair-wise genetic similarity according to the Dice similarity coefficients were calculated between taxa and used in the cluster analysis. The dendrogram obtained by the UPGMA clustering method revealed the genetic relationship of 19 taxa tested as shown in Fig. 3. The genera examined are roughly divided into two groups. The first group contains the genera *Boesenbergia* and *Scaphochlamys*, whereas the second group contains six species belonging to the genus *Kaempferia* as previously reported using taxonomic characters (Hussin et al., 2001) and isozyme information (Vanijajiva et al., 2003). According to the dendrogram, the first major group is divided into three subgroups. S1 and S2 are in the first, B5, B6, B10, B11, B2, B3 and B4 are in the second, while B1, B9, B7, and B8 are in the third subgroup. *B. pulcherrima* (B8) and *B. rotunda* (B10) are also classified in different subgroups as reported by Kress et al. (2002) using *matK* and ITS. Another major cluster is from genus *Kaempferia*. It is divided into two subgroups, (K1, K3 and K5) and (K2, K4 and K6) (Fig. 3). This finding was also observed by Kress et al. (2002) although different species of *Kaempferia* were used.

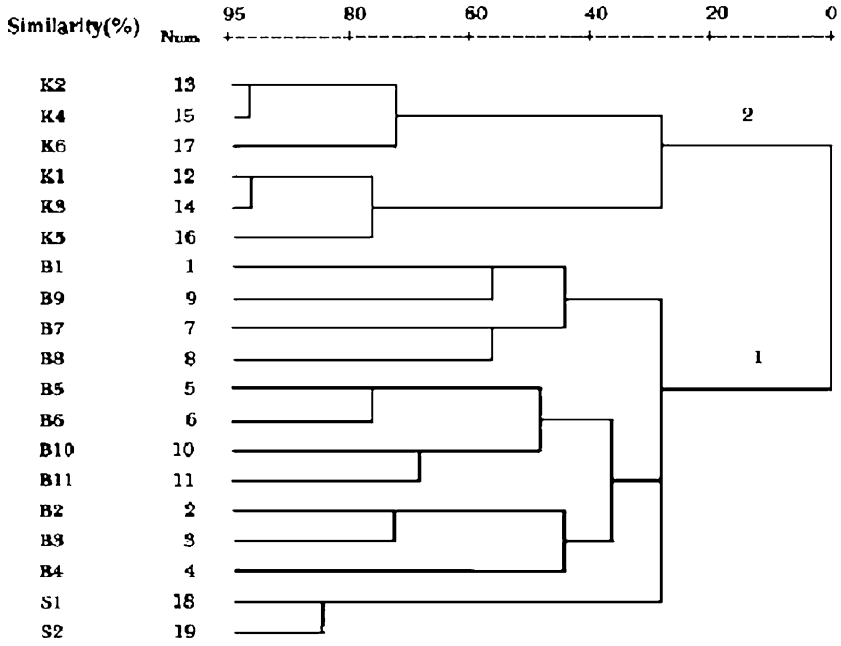

Fig. 3. Dendrogram based on UPGMA analysis of genetic similarity of 19 taxa obtained from RAPD, showing relationships among individual plants.

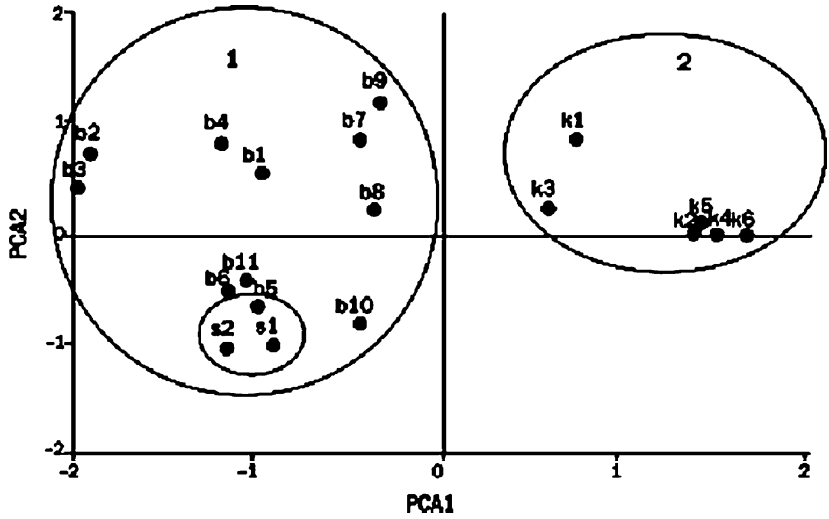

Fig. 4. Plot of PCA analysis of genetic distance of 19 taxa from RAPD, showing groups of individual plants.

The interspecific relationship among the 19 taxa by means of the first and second component of the principal components analysis shows two major groups (Fig. 4) which is in good agreement with the cluster analysis (Fig. 3). The members in the first major group are dispersed, they split into two distinct subgroups. The first subgroup includes S1, S2, B5, B6, B10 and B11, the second includes B2, B3, B1, B4, B7, B8 and B9. Another major group is formed by the genus *Kaempferia*. This group is clearly distinct from those of *Boesenbergia* and *Scaphochlamys*. The result agrees very well with the previous investigations on genera relationships of isozyme analysis information (Vanijajiva et al., 2003) and the taxonomic characters, for example, anther crest (Larsen et al., 1998) and inflorescences (Hussin et al., 2001) which says that *Boesenbergia* is closely related to *Scaphochlamys*. The detail grouping between *Boesenbergia* based on RAPD data, differs slightly from that of the isozyme (Vanijajiva et al., 2003) and the taxonomic characters such as labellum character (Sirirugsa, 1992) and inflorescences position (Larsen, 1993), possibly because both reflect only the coding regions of the genome while RAPD reflect both coding and non-coding regions of the genome. On the other hand, Kress et al. (2002) reported that *Boesenbergia* are polyphylectic and closer to *Cornukaempferia*, *Distichochlamys* and *Zingiber*. The discrepancy may be due to different approaches, in our case, we emphasized on 11 species of *Boesenbergia* and their relationship to *Scaphochlamys* and *Kaempferia* while Kress et al. (2002) studied all genera of the Zingiberaceae using only two species of *Boesenbergia* (*B. rotunda* 1 and 2 and *B. Pulcherrima*).

In conclusion, RAPD markers confirm genetic relationships among *Boesenbergia* and the genera previously reported. Therefore, RAPD is also a reliable method for estimating phylogenetic relationships since it reflects coding and non-coding regions of the genome and it could well be used in aiding identification as well as classification of the Zingiberaceae using more species in each genera.

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