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Original Article

Genetic variation of *Boesenbergia rotunda* (L.) Mansf. from Thailand based on essential oil compositions and internal transcribed spacer sequences

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Abstract

Boesenbergia rotunda (Zingiberaceae), an aromatic perennial herb, has been used in ethnomedicine as an antiflatulent, antidiarrheal, and antirheumatism agent. In this study, the genetic variation of three types of *B. rotunda* from Thailand, one wild type ('Krachai Pha') and two cultivars ('Krachai Ban' and 'Krachai Deang'), was studied by analyzing the essential oil compositions of their roots and rhizomes together with their internal transcribed spacer (ITS) sequences. The roots and rhizomes of 'Krachai Ban' and 'Krachai Pha' were rich in monoterpene hydrocarbons and oxygenated monoterpenes whereas the roots and rhizomes of 'Krachai Deang' were rich in oxygenated monoterpenes. The results of phylogenetic analysis based on the essential oil compositions and ITS sequences showed that 'Krachai Ban' was more closely related to 'Krachai Pha' than to 'Krachai Deang'. It should be concluded that three types of *B. rotunda* from Thailand might be potential sources for drug discovery.

Keywords: *Boesenbergia rotunda*, essential oil, ITS sequences, genetic variation

1. Introduction

Boesenbergia rotunda (L.) Mansf. is an aromatic perennial herb with a small and short rhizome and a fleshy fascicled root, that belongs to the family Zingiberaceae. It is widely cultivated and naturalized in Southeast Asia (Larsen & Larsen, 2006). The rhizome of *B. rotunda* was used in ethnomedicine as an antiflatulent, antidiarrheal, and antirheumatism agent (Chuakul & Boonpleng 2003; Maneenoon *et al.*, 2015; Neamsuvan, Tuwaemaengae, Bensulong, Asae, & Mosamae, 2012; Neamsuvan, Phumchareon, Bunphan, & Kaosaeng, 2016; Nontasit, Kanlayanapaphon, Mekanawakul, & Nualmangsar, 2015). In addition, Eng-Chong *et al.* (2012) reported that *B. rotunda* has several bioactive compounds that have strong biological activities such as anticancer, antimicrobial, antiviral, and antioxidant activities. In Thailand, three types of *B. rotunda* were recorded: 'Krachai Ban', 'Krachai Deang' and 'Krachai Pha' (wild type). 'Krachai Ban' is cultivated in several locations in Thailand, while 'Krachai Deang' and 'Krachai Pha' are naturally grown and rare type (Larsen & Larsen, 2006). Variation in morphological characteristics among the

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three types of *B. rotunda* such as the colour of the internal root and rhizome, midrib, laminar and leaf sheath, can be observed. In addition, each 'Krachai Pha' rhizome contains fewer small fascicled roots than does 'Krachai Ban'. The roots and rhizomes of 'Krachai Ban' are consumed as a spice in several Thai cuisines, and those of 'Krachai Deang' have been used as a magic plant in local religious rituals.

Essential oils are secondary metabolites that are produced by aromatic plants. They protect the plants through their antibacterial, antiviral, antifungal, and insecticide properties and also by reducing herbivores appetites for the plants (Mehdizadeh & Moghaddam, 2018; Nakatsu, Lupo, Chinn, & Kang, 2000). Therefore, essential oils have been widely used in the pharmaceutical, food, and cosmetic industries. Ample research has been devoted to studying genetic variation in plants using the similarities of essential oil compositions (Labra *et al.*, 2004; Tawfeeq, Culham, Davis, Reeves, & Michael, 2016; Tonk *et al.*, 2010; Vieira, Grayer, Paton, & Simon, 2001). For an individual plant species, chemical characteristics were less dependent on genetic variation than morphological characteristics (Ankanna, Suhrulatha & Savithramma 2012; Bhargava, Patel, & Desai, 2013; Mannheimer, 1988). As a result, essential oil compositions have been applied for the study of plant diversity.

To date, several DNA marker techniques have been shown to be reliable and valuable tools for the identification and evaluation of genetic diversity at various infrageneric levels, as they provide consistent results, irrespective of age, tissue origin, physiological conditions, environmental factors, harvest, storage, and processing of samples (Abdel-Mawgood, 2012; Heubl, 2010; Mondini, Noorani, & Pagnotta, 2009). Among several DNA marker techniques, the DNA sequencing technique is considered to be one of the most powerful tools to characterize species and to analyze phylogenetic relationships, population genetics, and evolutionary processes (Heubl, 2010). The internal transcribed spacer (ITS) region is a valuable genetic sequence that is useful for the study of phylogenetic and genomic relationships of plants at lower taxonomic levels (Forough, Shivaji, & Mallikarjun, 2018; Abdel-Mawgood, 2012). ITS sequences were applied to assess genetic variation in the infrageneric levels in the dicotyledonae genera; *Ficus*, *Lilium*, *Trigonella*, and monocotyledonae genera; *Saccharum*, *Zingiber* (Du *et al.*, 2014; Forough *et al.*, 2018; Ghada, Ahmed, Messaoud, & Amel, 2013; Kakani *et al.*, 2011; Thummajitsakul, Kaewsri, & Deetae, 2016).

Although there have been a number of previous studies on genetic variation in *Boesenbergia* species, no reports about genetic variation in the three *B. rotunda* types in Thailand have been recorded (Ngamriabsakul & Techaprasan, 2006; Techaprasan, Ngamriabsakul, Klinbunga, Chusacul tanachai, & Jenjittikul, 2006; Techaprasan, Klinbunga, & Jenjittikul, 2008; Vanijajiva, Sirirugsa, & Suvachittanont, 2005). Thus, the aims of this study were to evaluate the genetic diversity and phylogenetic relationships of three *B. rotunda* types including 'Krachai Ban', 'Krachai Deang' and 'Krachai Pha' on the basis of essential oil composition and ITS sequences. The results obtained from this study might provide useful information for the taxonomic study of *B. rotunda*, and for plant conservation, and might also serve as a genetic resource.

2. Materials and Methods

2.1 Plant materials

Fresh roots and rhizomes of three types of *B. rotunda* ('Krachai Pha', 'Krachai Ban' and 'Krachai Deang') were collected in the month of June to August 2015, from Chiangmai, Pathumthani, and Ratchaburi Provinces, Thailand, respectively. The plant samples were identified by Asst. Prof. Dr. Thaya Jenjittikul (Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand). The voucher specimens of all plant samples were deposited at the College of Pharmacy, Rangsit University, Thailand (voucher no. RSU 0053, RSU 0058 and RSU 0060). The living specimens were also cultivated.

2.2 Essential oil compositions and cluster analysis

2.2.1 Extraction of essential oils

The fresh roots and rhizomes of each plant sample (300 g) were washed with tap water, air-dried, cut into small pieces and ground. Each ground plant material was subjected to water distillation using a Clevenger apparatus for 3 hr. Essential oils were collected and stored at 4°C in air-tight containers before being analyzed by gas chromatography-mass spectrometry (GC-MS).

2.2.2 GC-MS analysis

GC-MS analysis of essential oils was performed using an Agilent 7890A gas chromatograph connected to a 5975C TAD inert XL EI/CI MSD over a DB-5 MS capillary column (30 m x 0.25 mm). Helium gas was used as the carrier gas at a flow rate of 1 ml/min. Each essential oil was diluted with ethanol (1:25 v/v). Next, 1 μL of the diluted essential oil was injected into the GC-MS machine using a GC 80 headspace autosampler in the splitless mode. The GC oven temperatures were programed as follows: starting at 60°C for 1 min, raised to 240°C at the rate of 3°C/min, and held at 240°C for 5 min. The temperatures of the GC injector and the GC-MSD interface were set at 180°C and 290°C, respectively. Electron impact ionization positive mode at 70 eV was acquired over the mass range of 40-650 m/z at a scan rate of 2.42 amu/sec. The total scanning time was 70 min.

2.2.3 Identification of essential oil components

Essential oil components were identified by comparing their mass fragmentation pattern with the Adams Essential Oil Mass Spectral library and the NIST05 Mass Spectral library. The amount of each oil component was determined using peak area measurement.

2.2.4 Statistical analysis

Hierarchical cluster with between-groups linkage method (SPSS Statistics version 18) was used to classify and group plant samples according to their chemical compositions for each essential oil.

2.3 ITS sequences and cluster analysis

2.3.1 Extraction of genomic DNA

A fresh young leaf of each plant sample was ground in liquid nitrogen with a mortar and pestle to obtain a fine powder. The genomic DNA from the powder was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

2.3.2 PCR amplification of ITS region

The ITS (ITS1-5.8S rDNA-ITS2) region was amplified using a pair of universal primers (Forward primer ITS5: GGA AGT AAA AGT CGT AAC AAG G and Reverse primer ITS4: TCC TCC GCT TAT TAT TGA GC) (Bertini *et al.*, 1999). For PCR amplification, 25 µl of a reaction mixture containing 1X amplification buffer, 2.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 unit of *Taq* DNA polymerase (Fermentas, USA), 0.1 µM of each primer (Operon Biotechnologies, Germany), and 1 µl of DNA template were prepared. PCR amplification was performed in a thermal cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 30 sec; a final extension at 72°C for 5 min; and a hold at 4°C. Next, 5 µL of each PCR product was separated by electrophoresis alongside a molecular weight marker (1 kb GeneRuler, Fermentas, Canada) on a 1.5% agarose gel in 1X TBE buffer. The gel was then stained with ethidium bromide and photographed using a UV transilluminator (Syngene, USA). The PCR products were purified using Qiaquick PCR purification kit (Qiagen, Germany) according to manufacturer's protocol and then submitted to Ward Medic Ltd., Part (Bangkok, Thailand) for DNA sequencing.

2.3.3 Phylogenetic analysis

All sequences were scored as 0 or 1 for the absence or presence of the same nucleotides, respectively. The similarity indices were calculated from the data that were generated using Dice similarity index coefficients (Nei & Li, 1979). A dendrogram was constructed based on the similarity matrix data using the unweighted pair group method with arithmetic averages (UPGMA), and was clustered by FreeTree software (Pavlicek, Hrda & Flegr, 1999). To evaluate the strength of the resulting branches, the bootstrap probabilities were calculated using 1,000 bootstrap resampling data with the FreeTree software.

3. Results and Discussion

3.1 Chemical compositions of the essential oils

The essential oils obtained from the water distillation of fresh roots and rhizomes of three types of *B. rotunda* from Thailand were clear and pale yellow with percent yields of 0.18-0.26%. The chemical compositions of each essential oil, as well as their peak area percentages, Kovats Indices (KIs) and percent yields, are shown in Table 1. A total of 54 compounds representing 97.83-99.22% of the total oil compositions were identified.

Oxygenated monoterpenes, including 1,8-cineole [14], linalool [17], camphor [20], α-terpineol [24], and geraniol [28], were found in the root and rhizome essential oils of all plant samples. Camphor [20] was the main component of the rhizome essential oil of 'Krachai Ban', and the root and rhizome essential oils of 'Krachai Pha' whereas *cis* β-ocimene [9], a monoterpene hydrocarbon, was the main component of the root essential oil of 'Krachai Ban'. The root and rhizome essential oils of 'Krachai Deang' were rich in oxygenated monoterpenes which was represented by 1,8-cineole [14].

These findings are in agreement with previous reports, as camphor was determined to be the main component in the rhizome essential oil of *B. rotunda* from Malaysia (Jantan *et al.*, 2001; Sukari *et al.*, 2008).

In addition, the results showed that the root and rhizome essential oils of "Krachai Deang" and 'Krachai Pha' are a promising source of 1,8-cineole and camphor, respectively. 1,8-cineole and camphor are used as flavouring agents in several fragrances and cosmetic products and were reported to have various biological activities such as antioxidant, anti-inflammatory and antimicrobial activities (Beer, Zagorchev, Draganova-Filipova, & Lukanov, 2017; Juergens *et al.*, 2003; Porres-Martínez, González-Burgos, Carretero, & Gómez-Serranillos, 2015; Şimşek, & Duman, 2017; Zuccarini, 2009). Therefore, "Krachai Deang" and 'Krachai Pha' should be conserved as potential sources for drug discovery.

3.2 Genetic variation based on essential oil compositions

Based on the essential oil compositions of the roots and rhizomes of the three types of *B. rotunda* analyzed in this study, 'Krachai Ban' and 'Krachai Pha' were categorized into the same cluster, while 'Krachai Deang' was clearly separated from the others (Figure 1). The root and rhizome essential oils of 'Krachai Ban' and 'Krachai Pha' had high amounts of monoterpene hydrocarbons (40.28-40.69% from the root and 24.43-29.63% from the rhizome) and oxygenated monoterpenes (55.46-57.61% from the root and 63.42-72.15% from the rhizome); therefore, they were classified into the same cluster. 'Krachai Deang' was completely separated from the others due to the presence of high amounts of oxygenated monoterpenes (79.86% from the root and 97.83% from the rhizome).

3.3 Genetic variation based on ITS gene sequences

The amplified ITS products of the three types of *B. rotunda* from Thailand contained approximately 760 bp. The number of nucleotides was 724, 711, and 684 for 'Krachai Ban', 'Krachai Pha' and 'Krachai Deang', respectively (Figure 2).

A dendrogram was constructed according to the UPGMA cluster analysis using Dice similarity coefficients. Based on the dendrogram, 'Krachai Ban' and 'Krachai Pha' were clustered together, while 'Krachai Deang' was clearly separated from the others, with 100% bootstrap support (Figure 3). Table 2 shows that the Dice similarity indices of three types of *B. rotunda* ranged from 0.60927 to 0.99881. 'Krachai Ban' and 'Krachai Pha' showed the highest genetic

Table 1. Essential oil components of the fresh roots and rhizomes of three types of B. rotunda

No	Compound name	KI*	Content (%)					
			'Krachai Ban'		'Krachai Deang'		'Krachai Pha'	
			Rhizome	Root	Rhizome	Root	Rhizome	Root
	Monoterpene hydrocarbons							
1	Tricyclene	926	0.31	0.36	-	-	0.34	0.57
2	α-Pinene	939	1.05	1.27	-	-	1.02	4.20
3	Camphene	954	6.88	6.34	-	-	6.82	16.02
4	β-Pinene	979	tr	0.23	-	-	-	0.19
5	Myrcene	990	0.70	1.40	-	-	-	0.54
6	α-Phellandrene	1002	-	-	-	-	-	0.09
7	*p*-Cymene	1024	0.18	-	-	4.87	-	0.20
8	Limonene	1029	2.66	2.39	-	3.60	-	3.11
9	*cis*-β-Ocimene	1037	17.26	**27.74**	-	-	16.25	7.25
10	*trans*-β-Ocimene	1050	-	-	-	-	-	7.83
11	γ-Terpinene	1059	0.26	0.22	-	-	-	0.18
12	1-(6,6-Dimethylbicyclo [3.1.0]hex-2-en-2-) ethanone	1063	0.08	-	-	-	-	-
13	α-Terpinolene	1088	0.26	0.32	-	-	-	0.50
	Oxygenated monoterpenes							
14	1,8-Cineole	1031	20.11	13.87	**65.87**	**67.89**	13.18	9.46
15	*cis* Linalool oxide	1072	tr	-	3.55	-	-	-
16	*E*,*E*-2,6-Dimethyl-3,5,7-octatriene-2-ol	1090	0.23	0.11	-	-		0.12
17	Linalool	1096	2.42	2.00	1.83	1.15	2.36	1.51
18	*Z*,*Z*-2,6-Dimethyl-3,5,7-octatriene-2-ol	1115	0.09	0.32	-	-	-	-
19	*endo*-Fenchol	1116	0.05	-	-	-	-	0.10
20	Camphor	1146	**25.78**	20.09	7.93	2.59	**41.12**	**22.39**
21	Isoborneol	1160	0.20	0.26	-	-	2.38	2.69
22	Borneol	1169	0.89	0.64	-	-	1.73	1.10
23	Terpinen-4-ol	1177	0.37	0.33	-	-	0.66	0.33
24	α-Terpineol	1188	1.46	1.20	6.54	2.04	1.40	1.46
25	Safranal	1196	-	0.15	-	-	-	-
26	Nerol	1229	tr	-	-	-	-	-
27	Carvone	1243	-	-	-	3.25	-	-
28	Geraniol	1252	11.83	16.28	4.49	2.93	9.32	18.45
29	p-Menth-8-en-1,2-diol	1268	-	-	7.61	-	-	-
30	p-Cymene-7-ol	1290	-	0.09	-	-	-	-
31	Geranyl acetate	1381	tr	0.11	-	-	-	-
	Sesquiterpene hydrocarbons							
32	β-Panasisene	1382	-	-	-	-	-	0.11
33	Neoisolongifolene	1416	-	-	-	-	-	0.14
34	α-Santalene	1417	-	0.17	-	-	-	-
35	Caryophyllene	1419	-	-	-	7.57	-	-
36	Z,E-Farnesene	1440	0.05	0.39	-	-	-	
37	α-Bulnesene	1509	-	-	-	-	-	0.07
38	7-epi-α-Cadinene	1592	-	-	-	-	-	0.09
39	Alloaromadendrane	1641	-	-	-	-	-	tr
	Oxygenated sesquiterpenes							
40	Nerolidol	1563	-		-	-	-	0.19
41	Caryophyllene oxide	1583	-	0.13	-	-	-	-
42	Globulol	1590	-		-	-	-	0.05
43	Geranyl 2-methylbutyrate	1596	0.05	0.13	-	-	-	
44	Germacrone	1693	-	0.08	-	-	-	
	Phenylpropanoids							
45	Phenethyl methyl ketone	1230	tr	0.07	-	-	-	-
46	Methyl 3-phenylpropanoate	1280	0.13	-	-	2.38	-	-
47	E-Methyl cinnamate	1378	5.87	1.64	-	-	-	-
	Others							
48	Benzaldehyde	960	0.07	-	-	-	-	0.06
49	Methyl benzoate	1074	tr	-	-	-	-	-
50	1,1,5-Trimethyl-1,2-dihydronaphthalene	1396	-	-	-	-	-	0.09
51	1,9-Tetradecadiene	1411	-	0.44	-	-	-	-
52	5,6-Dipropyldecan	1483	-	-	-	-	0.07	-
53	Hexadecene	1600	-	-	-	-	0.62	-
54	Octadecane	1800	-	-	-	-	0.73	-
	Total identified		99.22	98.79	97.83	98.28	97.99	99.10
	% Yields		0.26	0.22	0.21	0.19	0.20	0.18

*Kovats index (KI) is determined relative to n-alkanes (C6-C24) on a DB-5 MS column. tr < 0.05%; – not detected

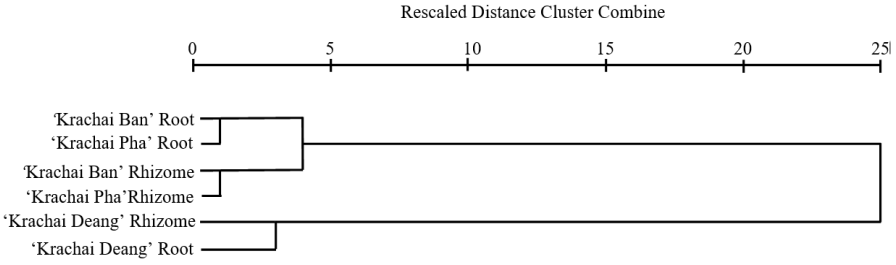

Figure 1. A dendrogram obtained from the cluster analysis based on essential oil compositions of three types of *B. rotunda*

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BR_P            ---------------TTTCCTCCGGGCCTATGATATGCTTAAA--CTCGGCGG-GTAGCC
BR_B            GTTCGTTTTTCTCCCGTTCCTCCGA-CTTATGATATGCTTAAG--CTCGGCAGTGTGGCC
BR_D            ----------------------------------GCTTAGGGGTACTGCGGGAAGATC
                                                   *****      * ** *      *
BR_P            ATTCGGGCTGCTGACTTGGGGCCACAATTAGATGGA------TGGACACGGCGCTTTCGT
BR_B            TCGC------CTGACTAGGGACCACAATTAGATGGAA-----TGGACACGGCGCTTTCGT
BR_D            ATTGTTTGAGAGAGCATAGAATGACGGATGATTGTGAACGTGTGAATGCGCCCCCTTTCT
                          *   *    **   *   **        ** *  ** * * **  *
BR_P            CCGATGTCGCAATCGCACGGGGTCTCTATCTAGGGCTCATCCCAAAACGACGAGGACGAT
BR_B            CCGATGTCTCAATCGCACGGGGTCTCTA----GGGCTCATCCCAAAACCACAAGGACAAT
BR_D            TTGCCCCCCCCCTGGTTGATGGGCATTGTTCGCCGCTCCTTGCGATCCTCCCTACTGAAG
                  *    * *  * *     ** *  *       **** *  * *  *  *       *
BR_P            GTTCTGTTCCCGCTCACGGCGACCAACGCCCATCGTGCCCGACGATTGCCGACTGCCCGC
BR_B            ATTATGATCCCGCTCACGGCCACCAACGCCCATCGTGCTCGACGATTGCAGACTGCCCGA
BR_D            AACAAAGTCCTAAGCA-GACGACCGACTCCCAGGGTACCC--------CGAGGCGCCCGA
                       ***    ** * * *** ** ****  ** * *        *     *****
BR_P            TCTTCGACCGACTGTGCCCTAGGGCACACGGGGCCAATTTCCGCGCTCACGCCAACCACG
BR_B            TCTTCCACCGACTGTGCCATAGGGCACAAGGGGCCAATTTCCGCGCTCACGCCAACCACG
BR_D            TATTTTTCAGATTTT-TCTGAATCTAATGAATCTCTATCATGGCTCTTGCATCTCTTACA
                * **   * ** * *  *  *    *        * **    ** **  *  *    **
BR_P            CATGGAGCGAAG-GCGACGAT-GTCATGAC--GCCCAAGCAGGCGTGCC-CTCGGCCA--
BR_B            CATGGAGAGAAG-GCGACGAT-GTCATGAC--GACCAAGCAGGCGTGCC-CTCGGCCA--
BR_D            AACATAAAGAAATGAGATAATTGGTGTGCTTGGTGTAAATTGCTGAACCACTTGATCTAT
                 *   *  ***  * **  ** *   **    *   **   *  *  ** ** *  *
BR_P            CAAGGCCTCGGGCACAACTTGCGTTCAAAGACTCAATGGTTCACGA---GATTCTGCAAT
BR_B            CAAGGCCTCCGGCACAACTTGCGTTCAAAGACTCAATGGTTCACGA---GATTCTGCAAT
BR_D            TAACTCTTTTAGCGCCCGTTGCCTTCGAGGCCATGG-GGCCCATGCTTCGCCTTCTTGGC
                 **  * *   ** *   **** *** * * *     **  ** *    *  *
BR_P            TCACACCAAGTATCGCATTTCACTACGTTCTTCATCGATGCAAGAGCCGAGATATCCATT
BR_B            TCACACCAAGTATCGCATTTCACTACGTTCTTCATCGATGCAACAGCCGAGATATCCACT
BR_D            CTCCTCGCCTTCGCCCCCTTCGCTCCTTGCGTGATTGCCGTGATTGCCCACATTTGCCCT
                   * *    *  * *  *** ** * * * * ** *  *  *  *** * ** * *  *
BR_P            GCCGAGAGTCATTTGATTCCGAAAAATCTCCGACGCATCGGGCTCCCTTGCGCACGCC--
BR_B            ACCGAGAGTCATTTGATTCCGAAAAATATCCGACGCATCGGGCTCCCCCGCGCACGCCTT
BR_D            CTGTGCCCTCAGTCGGTCC---AAGAGCTCGAAGACGGCGATCGCCGTCATCGATGGGCA
                        *** * * * *   ** *  **  *  *  **  * **       * *
BR_P            -GGGAGGCCGTCTGCTTCTG-AGTTCATTGTTCCTTAGTGCGGATCGCACCGA-GCTACG
BR_B            GAGGAGTCCGTCTGCTTCTG-AGTTCATTGTTCCTTAGTGCGGATCGCACCGA-GCTACG
BR_D            TTAGTCGCCGTGATCGCCAACAGAACATC-CTCATCATCGTCT-TCGTATTAGCACCACA
                   *   ****   *  *   **  ***   ** * *  *    ** *      * **
BR_P            GTCAATGCCCGCCGACATGGGGGGCAAGCAAGGAAAGGGGCGCATTCACACGTTCACAAT
BR_B            GTCAATGCCCGCCGACATGGGG----AGCAAGGAAAGGGGCACATTCACACGTTCACAAT
BR_D            TGCAGACCCTGTCCAATTG-------ATTGCGTCGTCTGACGCAACCG-ACGTGTCCATC
                  **   ** * * *  **       *    *      * * **  *  ****   **
BR_P            CATCCGTCATTCTATGCTCTCTCAA-CAATGATCCTTCC-GCAGGTTCACCT--ACGGAA
BR_B            CATCCGTCATTCTATGCTCTCTCAAACAATGATCCTTCCCGCAGGTTCACCCTAAGCGGA
BR_D            CAGCTA-CTTCTGCCACCTACTTATGCGAGGCCACCCCC--CGAGTTTAAGCATATCATA
                ** *   * *      *   ** *  * * *   *  **  *  *** *     *    *
BR_P            GACCTTGTTACGACTTTTTACTTCCAAATTTTTT------
BR_B            AACCTTGTTACA--CTTTTACTTCCATACTTCCACAGCAG
BR_D            AGCGCTGTAAAAA-----AACGGAGGAACTC---------
                  *  *** *         **      * *
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Figure 2. ITS sequences of three types of *B. rotunda*, BR_P : 'Krachai Pha'; BR_B : 'Krachai Ban'; BR_D : 'Krachai Deang'

similarity index (0.99881) whereas 'Krachai Pha' and 'Krachai Deang' showed the lowest similarity index (0.60927).

The dendrogram results were also correlated with the morphological characteristics. 'Krachai Ban' and 'Krachai Pha' have a small rhizome, green colour on the both sides of the lamina, and green midrib. 'Krachai Deang' has a large rhizome, a grayish green colour on the upper surface, a purple colour on the lower surface, and a purple midrib. However, the root and rhizome of 'Krachai Pha' and 'Krachai Deang' are yellowish orange to orange internally while 'Krachai Ban' is yellow internally. In addition, 'Krachai Pha' and 'Krachai Deang' have purple leaf sheaths while 'Krachai Ban' has a green leaf sheath. The important morphological characteristics of each cluster are shown in Table 3.

4. Conclusions

In this study, the genetic variation of three types of *B. rotunda* in Thailand, ('Krachai Ban', 'Krachai Deang' and 'Krachai Pha') was evaluated using the combined analysis of essential oil compositions and ITS sequences. The results indicated that 'Krachai Ban' was more closely related to 'Krachai Pha' than 'Krachai Deang'. This is the first report on the genetic variation of the three types of *B. rotunda* from Thailand. The results obtained from this study might provide useful information for plant conservation and taxonomic studies of *B. rotunda*, and as a genetic resource. Like earlier relative research works (Filho *et al.*, 2012; Rodrigues *et al.*, 2013), the essential oil compositions and molecular markers accompanying morphological characteristics of three *B. rotunda* types were implemented together to assure the coherent experimental results of their genetic diversity and phylogenetic relationships. The results indicated that the use of essential oil compositions together with molecular markers, as performed in this study, might be an alternative strategy for studying genetic variation at the infrageneric level.

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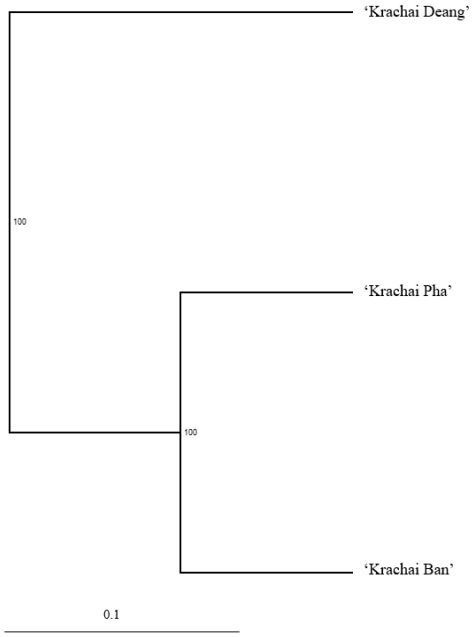

Figure 3. A dendrogram obtained from the cluster analysis based on ITS sequences of three types of *B. rotunda*

Table 2. Similarity matrix of three types of *B. rotunda* generated using Dice similarity coefficients

	'Krachai Ban'	'Krachai Pha'	'Krachai Deang'
'Krachai Ban'	1.00000		
Wild type	0.99881	1.00000	
'Krachai Deang'	0.61197	0.60927	1.00000

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Table 3. Important morphological characteristics of three types of *B. rotunda*

Morphological characteristics	'Krachai Ban'	'Krachai Deang'	'Krachai Pha'
Rhizome size (diameter)	0.8-1.0 cm	1.5-1.8 cm	0.8-1.0 cm
Root & rhizome colour			
External	Light brown	Orange brown	Light brown
Internal	Yellow	Orange	Yellowish orange
Leaf sheath colour	Green	Purple	Purple
Lamina colour			
Upper surface	Green	Grayish green	Green
Lower surface	Light green	Purple	Light green
Midrib colour	Green	Purple	Green

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