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Full Length Research Paper

Random amplified polymorphic DNA analysis of *Thunbergia laurifolia* Lindl. and its related species

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***Thunbergia laurifolia* Lindl. is an important herb in Thai folk medicine that has been widely used as an antidote, anti-inflammatory and antipyretic. However, counterfeits of *T. laurifolia* have been commonly found in Thai herbal markets and have arisen based on their similarity in appearance and shared common names. Flowers of plants belonging to *Thunbergia* spp., including *T. laurifolia, Thunbergia grandiflora* and *Thunbergia erecta*, look similar to each other, which often cause confusion when identifying these species. Moreover, there is another group of plants with names that share a similar common Thai name, "Rang Chuet", which includes a *Curcuma* sp., *Rinorea* sp., *Crotalaria spectabilis*, and *T. laurifolia*. In this study, we developed a random amplified polymorphic DNA (RAPD) method to facilitate the rapid detection of these medicinal materials. Out of 80 screened primers, nine gave clear and reproducible RAPD patterns. Among 164 amplified bands, 70 bands were polymorphic in six species. The dendrogram was constructed using unweighted pair group method with arithmetic averages (UPGMA), and the genotypes were differentiated into three main groups. The RAPD profiles of six species showed low similarity indices (0.1235 to 0.3137), whereas the genetic relationship was associated with the botanical characterization. These results indicate that RAPD is a useful tool for differentiating *T. laurifolia* from its related species.**

Key words: Acanthaceae, DNA fingerprint, molecular characterization, random amplified polymorphic DNA, *Thunbergia laurifolia.*

INTRODUCTION

Thunbergia laurifolia Lindl., which is a member of the Acanthaceae family, is commonly known as "Rang Chuet" in Thailand. It is a woody climbing plant that is native to South and Southeast Asia. The plant is traditionally used in detoxification, and as the first-aid treatment for poisoning from insecticides, ethyl alcohol, arsenic and strychnine (Thongsaard and Marsden, 2002). The leaves, stems and roots have also been used as anti-inflammatory agents and antipyretics for centuries (Tejasen and Thongthapp, 1980). The phenolic compounds of *T. laurifolia* have been shown to possess antioxidant activity (Oonsivilai et al., 2008), and an aqueous extract of the leaves have demonstrated hepatoprotective (Pramyothin et al., 2005), neuroprotective (Tangpong and Satarug, 2010) and hypoglycaemic effects (Aritajat et al., 2004).

In herbal markets, *T. laurifolia* is generally processed into a powder that is sold as an herbal tea or a capsulated preparation. However, the substitution or adulteration with other species is a common problem. Two species of herbaceous *Thunbergia*, *T. grandiflora* and *Thunbergia erecta*, are sometimes misidentified and used in place of *T. laurifolia*, as they have similar apparent morphology and phytochemical compounds. The flowers are shaped like a trumpet and are blue or pale blue in colour (Bor and Rhizada, 1982). Although iridoid glycosides and phenolic compounds have been reported in the leaves of *T. laurifolia* and *T. grandiflora* (Kanchanapoom et al., 2002; Ismail et al., 1996), limited

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

Plant name	Code	Family	Location (province)	Voucher No.
Thunbergia laurifolia Lindl.	TL	Acanthaceae	Bangkok	SS-0809101
			Nakhon Si Thammarat	SS-1009102
			Buri Ram	SS-1009103
			Prachin Buri	SS-1009104
			Nakhon Pathom	SS-0510105
			Nonthaburi	SS-0510106
			Uttaradit	SS-1010107
			Chiang Mai	SS-1110108
T. grandiflora Roxb.	TG	Acanthaceae	Bangkok	SS-1109201
			Prachuap Khiri Khan	SS-1209202
			Bangkok	SS-0510203
			Bangkok	SS-0810204
			Nakhon Pathom	SS-1210205
T. erecta (Benth.) T. Anderson	TE	Acanthaceae	Bangkok	SS-0809301
			Nakhon Pathom	SS-0510302
			Ubon Ratchathani	SS-1010303
			Chiang Mai	SS-1110304
Curcuma sp.	Cur	Zingiberaceae	Chachoengsao	SS-0909401
			Ratchaburi	SS-0710402
			Prachin Buri	SS-0710403
Rinorea sp.	Rin	Violaceae	Chachoengsao	SS-0909501
			Bangkok	SS-1209502
Crotalaria spectabilis Roth	CS	Papilionaceae	Nakhon Pathom	SS-0809601
			Bangkok	SS-1209602

research has been performed on *T. erecta*. Previous studies have shown that iridoid glycosides and phenolic compounds are commonly found in *Thunbergia* species (Jansen and Nielsen, 1989). The fact that *T. laurifolia* has been substituted and adulterated with other plants bearing the vernacular “Rang Chuet” in Thai, including *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*, is a matter of concern, as *Crotalaria spectabilis* contains poisonous pyrrolizidine alkaloids, which cause hepatotoxicity in mammals (Flores et al., 2009). Therefore, the accurate identification of *T. laurifolia* and its related species is necessary for safe and effective curative applications. In general, the approaches for identification depend on morphological and chemical analyses; however, some of these characteristics can be altered during the processing of the crude drug (Ma et al., 2000). Therefore, it is difficult to determine the botanical origins of crude herbs using morphological and chemotaxonomic studies.

Recently, various DNA-based methods have been successfully used for the pharmacognostic characterization of medicinal plants and herbal medicine, for the purpose of quality control and standardization (Zhao et al., 2006). Random amplified polymorphic DNA (RAPD) is considered a useful tool to distinguish between different botanical species due to its low cost and the good reliability of RAPD markers (Williams et al., 1990; Welsh and McClelland, 1990) and also because it is a relatively simple procedure, and previous sequence information is not required (Marieschi et al., 2009).

In the current study, we aimed to characterize *T. laurifolia* and its related species molecularly using the RAPD technique. The classification tree based on the DNA banding patterns of the plant species is also discussed.

MATERIALS AND METHODS

Plant materials

Whole *T. laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis* plants were collected from different locations in Thailand (Table 1). The plants were identified by Associate Professor Thatree Phadungcharoen (Department of

Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand), and they were compared with related herbarium specimens at the Forest Herbarium (Department of National Parks, Wildlife and Plant Conservation, Thailand). Voucher specimens were deposited in the Museum of Natural Medicines (Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand). Young leaves were collected from each plant and stored at -80°C until the DNA isolation.

DNA isolation and random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) amplification

A total of 100 mg of leaf tissue from each sample was frozen using liquid nitrogen and ground with a mortar and pestle to obtain a fine powder. The isolation of the total DNA from the powder was performed using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The quality of the genomic DNA was estimated after electrophoresis of an agarose gel and staining with ethidium bromide.

The DNA templates were added to 25 µl of PCR reaction mixture consisting of 1X amplification buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U of *Taq* DNA polymerase (Fermentas, Canada) and 0.4 µM random decamer primers (Operon Technologies, USA). The amplification was performed using a DNA thermal cycler (Bio-Rad, USA) with an initial denaturation of 2 min at 95°C, followed by 40 cycles of 45 s at 95°C, 1 min at 35°C, 2 min at 72°C and a final elongation of 5 min at 72°C. The amplification products were separated on 1% agarose gels and stained with ethidium bromide. The RAPD fragments were photographed using a UV transilluminator and analyzed with a gel documentation system (Bio-Rad, USA).

RAPD data analysis

The RAPD bands were scored as 0 or 1 for the absence or presence of bands, respectively. Only clear and reproducible bands were scored as 1. The molecular weights of the bands were estimated based on DNA markers (1 kb Plus, Invitrogen, USA). The similarity index was calculated from the data that was generated using Nei and Li's similarity index coefficient (Nei and Li, 1979). The dendrogram was constructed based on the similarity matrix data using the unweighted pair group method with arithmetic averages (UPGMA) clustering and FreeTree software (Pavlicek et al., 1999). To evaluate the strength of the resulting branches, bootstrap probabilities were calculated using 1,000 bootstrap resampling data with the aforementioned software.

RESULTS

RAPD was performed in triplicate using genomic DNA with 80 arbitrary decamers to test the reproducibility. Because each species that was collected from different localities showed the same pattern of RAPD profiles, an individual representative sample of each species was selected. Nine primers, OPA-03, OPA-04, OPA-07, OPA-10, OPN-02, OPN-10, OPN-12, OPN-16, and OPN-18, produced clear and reproducible polymorphic fragments in all six of the species, and all of the primers detected significant polymorphisms in the genomic DNA analysis. A total of 164 amplified bands with 70 polymorphic bands were scored from the population (Figure 1A-I).

The results demonstrated that different primers generated different fragment numbers and lengths. The size of the amplification products ranged from 0.4 to 4.9 kb (Table 2). The largest number of RAPD bands (25 bands) was detected using the OPA-04 primer (Figure 1B), whereas the smallest number of bands (8 bands) was generated with OPA-17 (Figure 1C). Unique RAPD bands were recognized in all of the investigated species. For *T. laurifolia*, the OPA-17 (Figure 1C) and OPA-18 (Figure 1D) primers consistently amplified an intense band at 2.8 and 2.6 kb, respectively. However, these bands were absent in the other species. Our results indicate that these fragments can be used as markers to distinguish between authentic *T. laurifolia* and its related species. Similarly, RAPD fragments of approximately 560 bp using OPN-10 (Figure 1F) and 1.2 kb using OPA-04 (Figure 1B) were only detected in *T. grandiflora* and *T. erecta*, respectively. Furthermore, a 710 bp fragment that was generated by the OPN-18 primer (Figure 1I) was a specific fragment in the *Curcuma* sp., OPA-18 (Figure 1D) produced a specific band of 730 bp in the *Rinorea* sp., and OPN-12 (Figure 1G) generated a unique band of 1.3 kb in *Crotalaria spectabilis*.

The pair-wise comparisons of the RAPD profiles that were based on both the shared and unique amplification products were used to generate a similarity index. Among the six species, the range of genetic similarity was from 0.1235 to 0.3137 (Table 3). The highest genetic similarity value was detected between *T. erecta* and *T. laurifolia* (0.3137), whereas the lowest similarity value was found between *C. spectabilis* and *T. laurifolia* (0.1235).

A dendrogram was constructed according to the UPGMA cluster analysis using Nei and Li's similarity coefficient. Based on the dendrogram, the six species were categorized into three major groups (Figure 2). The *Curcuma* sp. was individually isolated into the first group, whereas the three *Thunbergia* species, *T. laurifolia*, *T. grandiflora*, and *T. erecta*, were clustered into a second group. *T. erecta* was close to *T. laurifolia*, reflecting a narrow genetic diversity, whereas *T. grandiflora* was comparatively distant. The third group consisted of *Rinorea* sp. and *Crotalaria spectabilis*.

DISCUSSION

The confusion surrounding the identification of herbs in traditional medicine has been reported in many studies (Zhong et al., 2009; Bauer and Franz, 2010). Some of the various reasons that have been reported for such confusion have included herbs from multiple sources, region-specific herbs, similarities in appearance, complexity of processed products, and confusing nomenclature (Zhao et al., 2006). Specifically, the confusion regarding crude *T. laurifolia* preparations in herbal markets has been attributed to confusing nomenclature and the similarities to other plants in terms

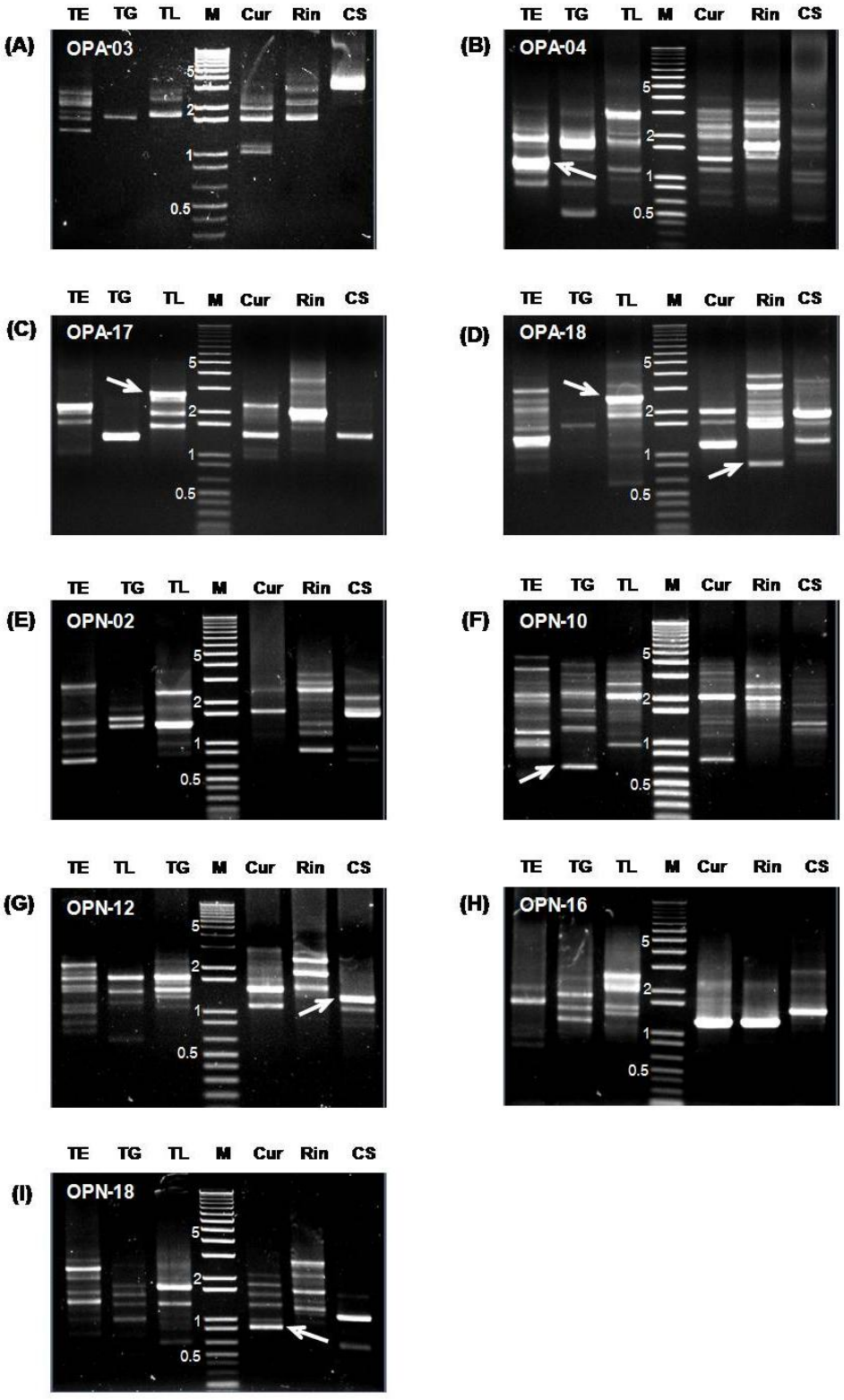

Figure 1. RAPD fingerprint of the six plant species obtained with the OPA-03 (A), OPA-04 (B), OPA-17 (C), OPA-18 (D), OPN-02 (E), OPN-10 (F), OPN-12 (G), OPN-16 (H) and OPN-18 (I) primers. TE: *Thunbergia erecta*, TG: *T. grandiflora*, TL: *T. laurifolia*, M: 1 kb Plus DNA marker (sizes shown in kb), Cur: *Curcuma* sp., Rin: *Rinorea* sp., CS: *Crotalaria spectabilis*. The unique fragments of each species are indicated with arrows.

Table 2. The sequence of the oligonucleotide primers used for the RAPD analysis and the banding patterns obtained from the six plant species.

Primer	Nucleotide sequence (5´ to 3´)	No. of bands	Size of bands	No. of polymorphic bands	No. of unique bands
OPA-03	AGTCAGCCAC	19	1040 - 3830	4	15
OPA-04	AATCGGGCTG	25	420 - 3760	10	15
OPA-17	GACCGCTTGT	8	1000 - 3770	4	4
OPA-18	AGGTGACCGT	19	510 - 4050	7	12
OPN-02	ACCAGGGGCA	16	590 - 3450	8	8
OPN-10	ACAACTGGGG	22	490 - 4890	16	6
OPN-12	CACAGACACC	20	580 - 3370	6	14
OPN-16	AAGCGACCTG	13	670 - 2660	5	8
OPN-18	GGTGAGGTCA	22	530 - 2700	10	12

Table 3. Similarity matrix of the six plant species generated using Nei and Li's similarity coefficient.

Species	Code	TE	TG	TL	Cur	Rin	CS
Thunbergia erecta	TE	1.0000					
T. grandiflora	TG	0.2222	1.0000				
T. laurifolia	TL	0.3137	0.3095	1.0000			
Curcuma sp.	Cur	0.2245	0.2000	0.1522	1.0000		
Rinorea sp.	Rin	0.2056	0.2247	0.2574	0.2474	1.0000	
Crotalaria spectabilis	CS	0.2069	0.2029	0.1235	0.1558	0.2791	1.0000

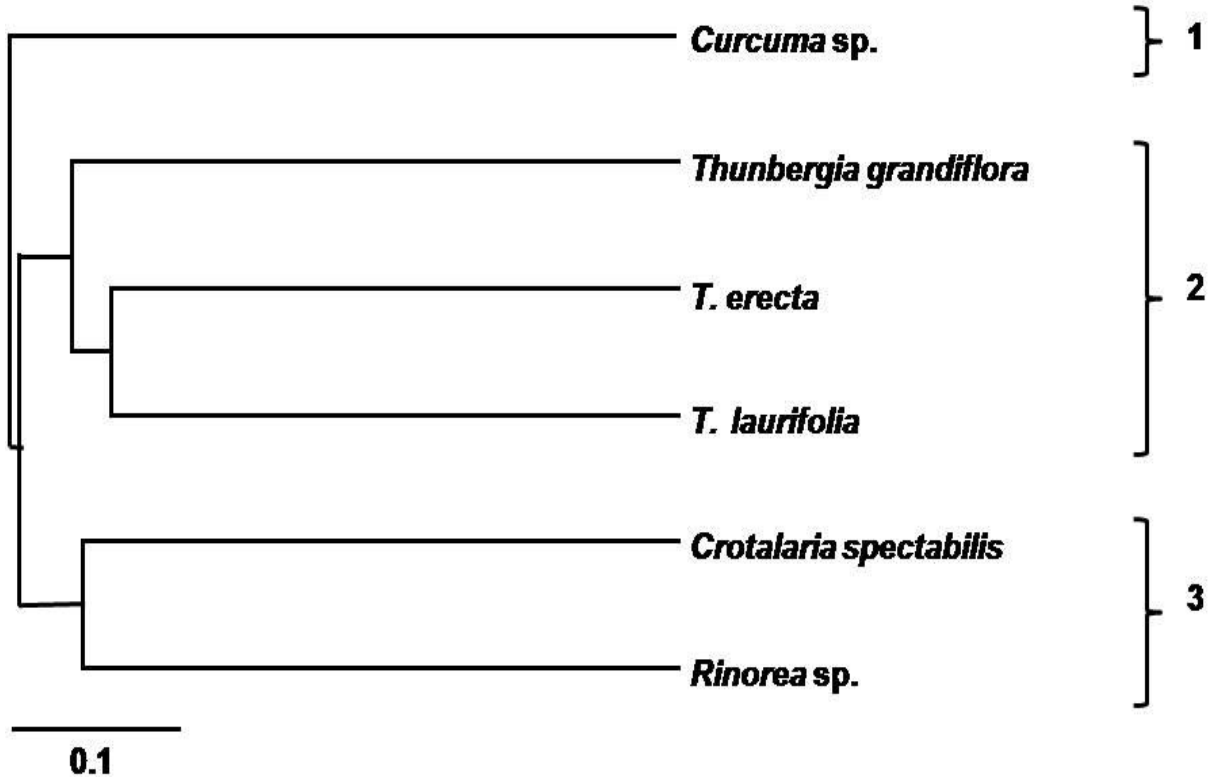

Figure 2. Dendrogram produced by UPGMA cluster analysis of RAPD data showing the genetic relationship among the six plant species. The similarity scale is indicated at the bottom left corner.

of appearance. However, curative effects of the substitutes or adulterants have not been reported. Moreover, the mistaken use of *C. spectabilis* in place of *T. laurifolia* can increase the risk of hepatotoxicity. Therefore, the correct identification and quality assurance of the starting material is an essential prerequisite to ensure the reproducible curative effects of herbal medicines.

RAPD analysis has been widely used to differentiate between many medicinal species and their close relatives or adulterants, including *Derris* spp. (Sukrong et al., 2005), *Desmodium* spp. (Irshad et al., 2009), *Encephalatos* spp. (Prakash and van Staden, 2008), and *Phyllanthus* spp. (Manissorn et al., 2010). The advantages of this technique include its speed, simplicity and the requirement for only small amounts of DNA (Mahmood et al., 2010). Indeed, RAPD analysis has been successfully used for taxonomic and systematic classification, and phylogenetic or genetic diversity studies in plants (Neog et al., 2010). Although RAPD markers have few disadvantages, some of the problems regarding the reproducibility of RAPD data and scoring errors have been addressed (Skroch and Nienhuis, 1995). As the standardization of DNA isolation techniques and PCR reaction conditions are keys in limiting errors, the use of intense staining, clearly resolvable bands and unvaried DNA samples have been shown to provide consistent results (Aruna et al., 1993).

In the current RAPD study, all six plants, *Thunbergia laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*, were differentiated from each other based on unique band patterns that were obtained after RAPD amplification. In addition, polymorphic fragments were used to distinguish among these studied species: nine primers produced 70 polymorphic bands that were essential to the construction of the dendrogram and differentiated among the six species. Using OPN-12 (Figure 1G), the intense fragments were approximately 1750 bp in *T. laurifolia*, *T. grandiflora* and *T. erecta*. Matching bands of similar size were not observed in the other species; therefore, these monomorphic bands were useful to characterize the species in the genus *Thunbergia*. However, patterns from other primers indicated the presence of genetic variability, which was also beneficial for identification of the different *Thunbergia* species.

The cluster analysis using RAPD sorted the six species into three main groups, which strongly correlated with the parents. The first group was comprised of *Curcuma* sp., which was the only monocotyledon plant in this study and was clearly separated from the other species based on molecular phylogeny. In the second group, *T. erecta* and *T. laurifolia* were clustered together, with a similarity value of 0.3137 and were comparatively separated from *T. grandiflora*. Our results contrast with those of a previous study, which has reported the comparison of the *trnT-trnL*, *rpl-16* and *rps-16* sequences in Thunbergioideae species and had identified a close relationship between *T. laurifolia* and *T. grandiflora* (Borg and McDade, 2008). However, the similarity value that we calculated from the RAPD data between *T. laurifolia* and *T. erecta* (0.3137) was similar to the value of *T. laurifolia* and *T. grandiflora* (0.3095). This result indicates the close genetic relationship of *Thunbergia* species compared with other species. Although *Rinorea* sp. and *Crotalaria spectabilis* are classified in different botanical families, the plants were genetically placed together in the third group. The closeness of the *Rinorea* sp. and *Crotalaria spectabilis* confirmed a previous report that grouped the plants in the rosid clade (Wang et al., 2009). The arrangement of these six plants in this dendrogram is relatively correlated with the plant classification and floral taxonomy following botanical principles.

Our study demonstrated that *T. laurifolia* was successfully distinguished from its related species based on their molecular signatures. The results affirm RAPD analysis as a technique that is able to examine the phylogenetic relationship of different plant species. Additionally, sequence characterized amplified regions (SCARs) can be further developed to identify specific and sensitive DNA markers.

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