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## **RAPD Technique Identifies Subtypes of *Vatica diospyroides* Symington, a Critically Endangered Medicinal and Fragrant Plant in the Dipterocarpaceae**

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### **ABSTRACT**

*Vatica diospyroides* Symington has two distinct commonly known subtypes: The SS and LS forms distinguished by the color of petals and cotyledons. These subtypes may also differ in their contents of the valuable products for human health-therefore it is important to develop and share a reliable method of subtype identification. The Random Amplified Polymorphic DNA (RAPD) technique was applied with 3× and 5×CTAB (Cetyltrimethyl ammonium bromide) extraction procedures, the concentrations of NaCl and DNA templates were varied and 10-mer random woody primers were screened to find distinguishing genetic indicators. The best yields of SS and LS DNA (412.8-578.4 µg g<sup>-1</sup> FW) were found with phenol: Chloroform: Isoamyl alcohol (25:24:1) followed by 5×CTAB buffer. Five effective primers (OPU5, OPU8, OPU9, OPU14 and OPU15) gave a total of 18 DNA bands (200-2500 bp), when using 1.5 mM NaCl and 200 ng µL<sup>-1</sup> DNA template in PCR conditions. Of the bands, 12 were evidently polymorphic. The primer OPU9 produced 1 polymorphic band (220 bp) for LS and 1 monomorphism (at 500 bp). With OPU15, a total of 4 bands were observed: The markers at 320, 480 and 520 bp specify the LS form, while at 900 bp the band is shared by both subtypes. In conclusion, the *V. diospyroides* subtypes distinguished by different color petals and cotyledons, are also genetically differentiated: The phenotypic differences are not environmentally induced. These initial results encourage pursuing genomics based phylogenetic analyses of *V. diospyroides*.

**Key words:** Flower, fruit, RAPD, variety, *Vatica diospyroides*

### **INTRODUCTION**

Plant genera in the family Dipterocarpaceae, such as *Dipterocarpus*, *Hopea* and *Vatica*, are important sources of active ingredients for cardiovascular prevention (Zain *et al.*, 2011). These plants are increasingly screened in cytotoxicity assays, because their stems contain resveratrol derivatives known to be curative active ingredients (Seo *et al.*, 1999; Ito *et al.*, 2003; Atun *et al.*, 2008). Two main valuable resveratrol derivatives have been reported in the genus *Vatica*: Vaticanol series was extracted from *V. rassak* (Ito *et al.*, 2003) and Vaticaphenol A was extracted from *V. diospyroides* Symington (Seo *et al.*, 1999). These derivatives had efficacy against some human cancer cell lines. A strongly fragrant dipterocarp endemic in peninsular Thailand,

*V. diospyroides* is described in monotypic taxon (Pooma, 2002) and has two subtypes (Srisawat *et al.*, 2012b). The Small Size (SS) subtype has narrow leaves and 1.8 pg DNA content; the Large Size subtype (LS) has wide leaves and 2.2 pg DNA content. These subtypes can be distinguished using specific characteristics of leaf morphology and flow cytometry (Srisawat *et al.*, 2012b) and also differ by the color of flowers. The SS trees have pink-white flowers, whereas yellow-white flowers are found in LS trees. The two subtypes can be reasonably expected to differ in the amounts and compositions of potential active ingredients in their extracts and methods to reliably identify the subtype are needed for such drug discovery studies. Effective but low cost genomic techniques to reliably discriminate the subtypes should be investigated.

Although, rapid and accurate detection of plant varieties has been reported by combinations of morphology assessment and flow cytometry, the molecular marker techniques are the reference methods used to assure the preciseness of those other methods (Srisawat *et al.*, 2012a). There are many potential molecular marker types and genomic techniques that can be applied to plant varieties, such as Amplified Fragment Length Polymorphisms (AFLP), Restriction Fragment Length Polymorphisms (RFLP), Simple Sequence Repeats (SSR) and Random Amplified Polymorphic DNA (RAPD) technique (Young, 2000). The RAPD technique has been common in preliminary comparisons to morphological patterns of various plant species (Kaewmuangmoon *et al.*, 2010; Sheidai *et al.*, 2010). In the family Dipterocarpaceae, PCR amplification of genomic DNA using random primers was used to discover unique genetic polymorphisms of some species in the genera *Hopea* and *Shorea* (Rath *et al.*, 1998) and in one species of *Vatica guangxiensis* (Li *et al.*, 2002). Unfortunately, the *V. diospyroides* species has not been evaluated genetically to date. Knowledge of genetic observable characteristics can be used for subtyping trees of *V. diospyroides*, possibly helping to find unique curative active ingredients in a subtype. In the present study, RAPD markers were sampled to discover DNA polymorphic patterns that distinguish between the subtypes of *V. diospyroides*, with a view to support such precise subtyping and consequently drug discovery work on these trees.

## MATERIALS AND METHODS

**Plant materials:** Natural *V. diospyroides* symington trees were labeled according to their subtype at the Nong Thung Thong non-hunting area, based on earlier morphometric and flow cytometric subtype identification (Srisawat *et al.*, 2012b). To extract genomic data in the laboratory, young leaves of selected plants were sampled. All leaf samples were surface-sterilized by soaking in tap water with disinfectant (commercial detergent), then rinsed with sterile distilled water. The cleaned samples were stored at -80°C prior to DNA extraction. The confirmed specimens (Collector number T. Srisawat 001 and 002) were deposited in the Herbarium of Queen Sirikit Botanic Garden (QBG) Maerim, Chiang Mai, Thailand. Flowers and fruits were picked and their colors compared for further confirmation of the subtypes.

**DNA extraction and purification:** The DNA extraction was done according to the protocol of CTAB (Cetyltrimethyl ammonium bromide) method (Doyle and Doyle, 1990), here modified for optimal DNA yield and purity. The frozen leaf samples were crushed in liquid nitrogen prior to transfer to 800 µL preheated 3×CTAB extraction buffer [0.1 M Tris-HCl pH 8.0, 0.5 M EDTA (Ethylenediaminetetraacetic acid) pH 8.0, 0.5 M NaCl, 3% CTAB, 2% PVP-40 (polyvinylpyrrolidone) and 2% β-mercaptoethanol]. The mixture was heated to 65°C for 30 min in a water bath and briefly centrifuged to precipitate cell debris. The contaminated protein in

supernatant was removed by a half volume of phenol and (phenol: Chloroform: Isoamyl alcohol) (25:24:1). After centrifugation and keeping the supernatant, an 0.1 volume of 3 M sodium acetate (pH 5.2) was added to the supernatant and subsequently two-fold volumes of ice-cold absolute ethanol were added to precipitate DNA. The precipitated DNA was washed with ice-cold 70% ethanol and air dried. The dried DNA pellet was redissolved in 25  $\mu$ L TE buffer containing RNase A to eliminate RNA contamination. In order to remove carbohydrates or phenolic compounds from purified DNA, it was mixed with 0.1 volume of 5% CTAB in 0.5 M NaCl and centrifuged at 8,000 rpm for 10 min. The DNA-CTAB complex pellet was dissolved with 200  $\mu$ L of 1.2 M NaCl and DNA was re-precipitated with 500  $\mu$ L of ice-cold absolute ethanol. The mixture was incubated in  $-20^{\circ}\text{C}$  for 1 h and centrifuged at 10,000 rpm for 10 min. The precipitated DNA was washed with ice-cold absolute ethanol, air dried and subsequently redissolved in TE buffer. The concentration and purity of DNA were measured with a Hitachi U-2900 Spectrophotometer (Hitachi High-Technologies Corp., Tokyo Japan). The DNA solution was diluted to 100-300 ng  $\mu\text{L}^{-1}$  for PCR amplification.

**PCR amplification and gel electrophoresis:** Ten random primers that can amplify reproducible and clear DNA bands of woody plants were selected. Six effective primers (OPU) of the family Dipterocarpaceae (Rath *et al.*, 1998) and four effective Primers (P) of another woody aromatic species, *Aquilaria* sp. in the family Thymelaeaceae (Panichayupakaranant *et al.*, 2006) were redesigned and prepared. Their sequences (5'-3') are shown in Table 1.

For Polymerase Chain Reaction (PCR), the 25  $\mu$ L reaction mixtures consisted of 4  $\mu$ L DNA template, 8  $\mu$ L ultra water, 0.5  $\mu$ L primer and 12.5  $\mu$ L 2 $\times$  blue mix DNA polymerase master mix (RBC Bioscience corp., Taiwan). The negative control consisted of all reagents except the DNA template. The PCR amplification was tuned by varying concentrations of  $\text{MgCl}_2$  (1.5-4.0 mM) and DNA template (100-300 ng) following the method of Rath *et al.* (1998). The amplification reaction was performed in a Multigene Thermal Cycler TC 9600 (Labnet International Inc., NJ USA). The program for PCR amplification was as follows; 1 cycle of initial pre-denaturation ( $95^{\circ}\text{C}$ , 2 min); followed by 30 cycles of denaturation ( $95^{\circ}\text{C}$ , 1 min), annealing ( $37^{\circ}\text{C}$ , 3 min) and extension

Table 1: Counts of mono and polymorphic RAPD bands and resulting percentages of polymorphism among *V. diospyroides* subtypes SS and LS, the determinations used OPU (Dipterocarpaceae) and P (Thymelaeaceae) primers

Random primers (5'-3')	DNA bands of SS		DNA bands of LS		Percentage of polymorphism
	Monomorphic	Polymorphic	Monomorphic	Polymorphic	
P1: TGGGCGCGCA	0	0	0	0	0.00
P2: GGAGCAGGCC	0	0	0	0	0.00
P3: CAGGGAGGCC	0	0	0	0	0.00
P4: GGACCGCCTG	0	0	0	0	0.00
OPU4: ACCTTCGGAC	0	0	0	0	0.00
OPU5: TTGGCGGCCT*	1	0	1	2	50.00
OPU8: GGCGAAGGTT**	0	0	0	2	100.00
OPU9: CCACATCGGT***	1	0	1	1	33.33
OPU14: TGGGTCCCTC**	0	0	0	4	100.00
OPU15: ACGGGCCAGT***	1	0	1	3	75.00
Total	3	0	3	12	66.66

\*Primer produced weak DNA bands in both SS and LS types, \*\*Primers produced only LS DNA bands, \*\*\*OPU9 and OPU15 produced the best apparent DNA bands for monomorphic and polymorphic bands of both SS and LS types

(72°C, 2 min) and final extension (72°C, 1 min). The amplification and VC 100 bp DNA Ladder markers were subjected to gel electrophoresis in 1.5% (w/v) agarose gel, with 0.5× TBE buffer and at 70 volts for 2 h. The gels were stained with SYBR@safe (Invitrogen Life Technologies Corp., NY USA) and imaged following UV Gel Documentation (DNR Bio-Imaging Systems, Jerusalem Israel).

## RESULTS

**DNA extraction and purification:** The extraction and purification of DNA from young leaves of both *Vatica diospyroides* Symington subtypes (SS and LS form) was performed according to method of Doyle and Doyle (1990). However, the DNA extracted by this method was not considered good enough to proceed to the next steps of RAPD-PCR because of poor DNA quantity and quality (data not shown). Moreover, the DNA pellet appeared brownish in color due to contaminating phenolic compounds. Thus, phenol extraction was further added as a step in the process, to eliminate the contaminating proteins and in the final step of DNA extraction, 5×CTAB extraction was repeated to purify the DNA of phenolic compounds or contaminating carbohydrates. Based on this process, the extracted DNA of the SS and LS types appeared as a yellowish-white DNA pellets with DNA concentrations of about 412.8-578.4 µg g<sup>-1</sup> FW and an OD ratio range of 1.5-1.6 (OD<sub>260</sub>/OD<sub>280</sub>). This DNA now had sufficient quality and quantity for use in PCR amplification. It was experimentally observed that the RAPD-PCR products from these DNA samples were of high enough quality for amplification and gave obvious polymorphic bands.

**PCR amplification, gel electrophoresis and distinction of tree types:** The amounts of DNA and concentrations of MgCl<sub>2</sub> were varied to optimize the PCR amplification. It is interesting to note that 100-300 ng of DNA template and 1.5-4.0 mM MgCl<sub>2</sub> produced detectable amplification products. Good RAPD profiles were obtained with 200 ng DNA template and 1.5 mM MgCl<sub>2</sub> in 25 µL reaction mixtures. The results revealed that 12 bands out of the total 18 of *V. diospyroides* DNA were polymorphic (Table 1) when using OPU5, OPU8, OPU9, OPU14 and OPU15 as primers. These results thus confirm effective primers for a dipterocarp species, for which there is no prior similar genetic data.

Based on the uniqueness of amplification, the five effective primers can be used for verifying the subtype. The best RAPD profiles for these subtypes were obtained with primers OPU9 and OPU15, are shown in Fig. 1a. Interestingly, in the case of OPU15, only LS subtype showed polymorphic bands at 320, 480 and 520 bp: These are markers for LS subtype. The other bands located at 900 bp showed monomorphic DNA patterns, the bands being shared by both subtypes. These bands are absent in both subtypes when using OPU9 primer. With this primer, the LS form had a polymorphic band at 220 bp and the subtypes shared a (monomorphic) band at 500 bp. The evident polymorphic bands indicate genetic differentiation of the LS and SS subtypes of *V. diospyroides*. On the other hand, the monomorphic DNA bands of LS analyzed by OPU9 and OPU15 showed higher PCR-product intensity than in SS.

Using other effective primers, OPU5, OPU8 and OPU14, although, it was also possible to distinguish between the subtypes, the DNA bands had poor polymorphic band quality at 600 bp, 300 and 1500 bp and 1200 and 2500 bp (data not shown). The remaining primers, OPU4 and Thymelaeaceae primers, produced no clear bands (Table 1). It is possible that some nucleotides in these primers might not be complementary to random target sites of *V. diospyroides* genome.

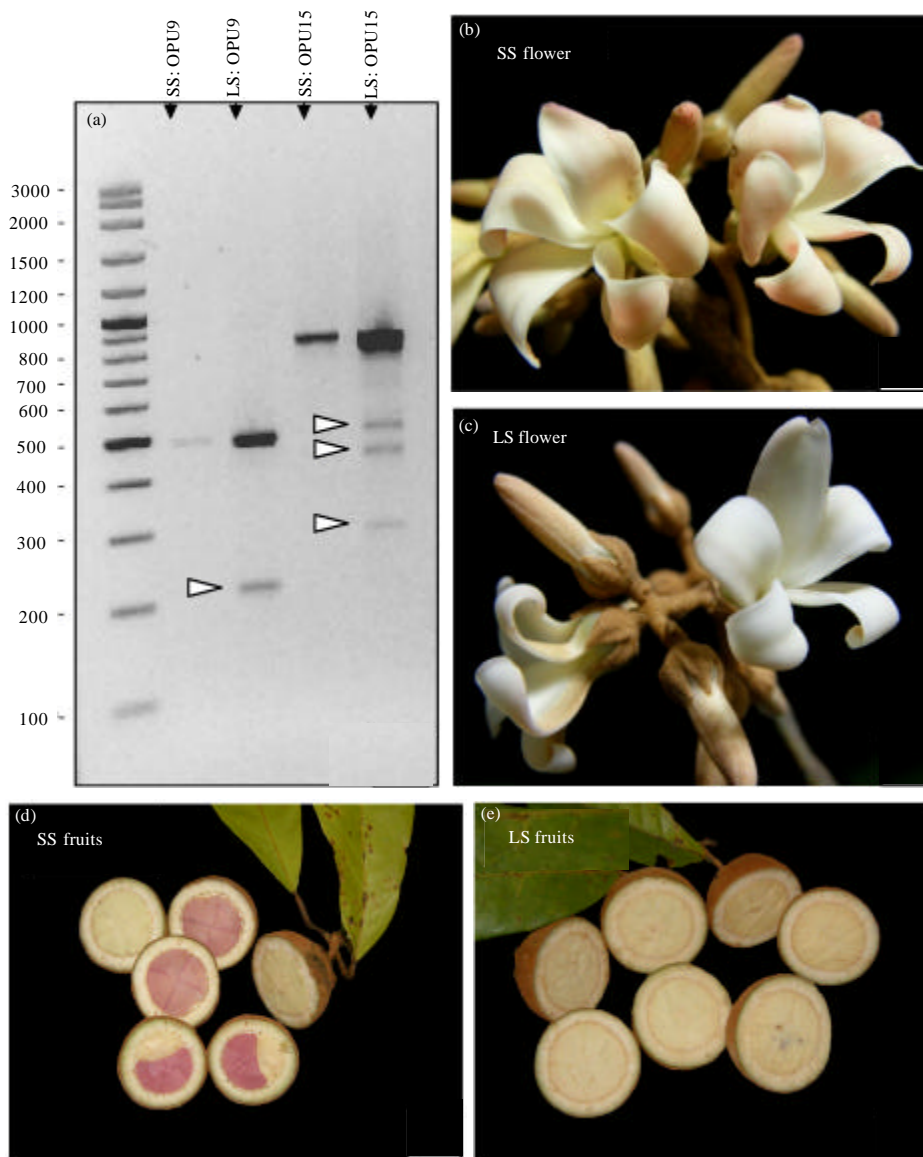


Fig. 1(a-e): Discrimination of SS and LS forms of *V. diospyroides* by various means, the discriminating unique polymorphic bands in RAPD profiles, generated with primers OPU9 and OPU15 are indicated by white arrows (a), pink-white and yellow-white petals of flowers enable discrimination (b and c), as do purple or yellowish seed colors, the latter associated with yellowish cotyledons (d and e)

## DISCUSSION

Plants in the family Dipterocarpaceae, as other woody plant species, can release phenolic compounds during crushing and extraction of tissue samples (Rath *et al.*, 1998; Sathish and Mohankumar, 2007). Contamination of extracted DNA by phenolic compounds often gives a brown tint to DNA samples that should be white (Srisawat *et al.*, 2012a). In the present study, the purified

DNA was brownish due to contamination by tannin or phenolic substances, attached in the DNA helices: Rajaseger *et al.* (1997) reported similar contamination by phenolic compounds during DNA extraction from *Ixora* sp. The present work has demonstrated the usefulness of phenol: Chloroform: Isoamyl alcohol solutions and 3× and 5× CTAB buffer to delimit the phenolic compound reactions in the DNA prior to RAPD-PCR amplification. The present modified DNA extraction and purification gave final DNA pellets with sufficient amount of purified DNA and compared favorably to other methods used in the Dipterocarpaceae (Rath *et al.*, 1998).

For PCR amplification and gel electrophoresis, various conditions such as amounts of DNA template and concentrations of MgCl<sub>2</sub> are known to greatly influence quality and quantity of RAPD profiles and polymorphisms. Comparison of different DNA amounts and MgCl<sub>2</sub> concentrations in amplifying polymorphic bands from the two *V. diospyroides* subtypes showed that a reaction mixture containing 200 ng DNA template and 1.5 mM MgCl<sub>2</sub> was very efficient, resulting in a number of observable polymorphic bands. This resembles results obtained by Rath *et al.* (1998). The detected 12 polymorphic bands of both subtypes were from the most effective dipterocarp primers namely OPU5, OPU8, OPU9, OPU14 and OPU15 (Rath *et al.*, 1998). The bands were in the size range from 220-2500 bp, differing from the range previously reported for the other genera in dipterocarp species (Rath *et al.*, 1998).

Interestingly, the best RAPD profiles were found when using OPU9 or OPU15 as relative primer, resulting in 4 conspicuously polymorphic bands. These effective primers have been used on PCR amplification of genomic DNA of various species in dipterocarp genera such as *Hopea* and *Shorea* (Rath *et al.*, 1998). On the other hand, Li *et al.* (2002) successfully reported on RAPD analysis with the *V. guangxiensis* species resulting in more polymorphic bands than those obtained from another species. However, they did not provide the sequences of their primers, in contrast to the report by Rath *et al.* (1998). In relation to the present study, a complementary primer could be designed to match the DNA strand and/or the primer length could be increased which would enable annealing at more than one complementary site within the genome of *V. diospyroides* (Bock, 1997). The use of modified OPU primers might significantly contribute to obtaining highly reliable genetic differentiation between plant species of dipterocarpaceae family. However, based on the current results, the use of OPU9 and OPU15 with PCR amplification, to distinguish between *V. diospyroides* subtypes, is a reasonable initial approach.

As described above, *V. diospyroides* could be separated distinctly into two groups according to differences in the presence of polymorphic and monomorphic bands and the grouping agreed with the results of leaf morphology and flow cytometry analyses (Srisawat *et al.*, 2012b). Observations have also revealed a high variety of different colors of flowers and fruit-cotyledons of *V. diospyroides* (Fig. 1). These discriminating characteristics have not been described previously, though the classical and Scanning Electron Microscopic taxonomies have been documented extensively (Smitinand, 1966; Ashton, 1998; Poorna and Newman, 2001; Srinual and Thammathaworn, 2008). These variations within a species should be due to genetic factors, because most environmental factors affect the shape, color and size of leaves (Wilson *et al.*, 1998; Campey *et al.*, 2000), indicating that this species is actually a composite of species. In order to identify conventional heredity, a self-cross breeding investigation among these tree subtypes should be performed in the blossoming time. Unfortunately, sample collection was affected by the fact that after a severe and long drought in Thailand (2009-2010), blooming of *V. diospyroides* during its blossoming period (February to April) was not normal (Srisawat *et al.*, 2012b). It was only possible to sample two LS and two SS trees of *V. diospyroides*, because of the requirement to

produce flowers and fruit for subtype confirmation. Due to the minimal sampling, no statistical methods could be applied in the present study. This circumstance illustrates the need for subtype identification not based on phenotypic characteristics of, for example, flowers.

In conclusion, the DNA polymorphisms indicate genetic variability that differentiates between the subtypes of *V. diospyroides*. The polymorphisms were apparent with OPU5, OPU8, OPU9, OPU14 and OPU15 primers in RAPD analysis. The most effective primers for discriminating between the subtypes of *V. diospyroides* were OPU9 and OPU15. This suggests that methods based on molecular DNA markers can supplement or replace assessments of plant coloring or characterizing. A systematic phylogenetic study of *V. diospyroides*, based on molecular markers of DNA, seems justified in light of these preliminary results by RAPD analysis. The present study is the first report on successfully detecting differentiating genetic polymorphisms between subtypes of *V. diospyroides* trees by a biomolecular method.

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