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Research Article
Genetic-relatedness of Tuba Plants from Peninsular Malaysia and Quantitative Analysis of their Rotenone

Norhayu Asib and Dzolkhifli Omar

Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Abstract

Background and Objective: Tuba plants is a small flowering shrub originating in the humid rainforest of Malaysia. Tuba plants is known to contain the chemical rotenone, which is have an insecticidal properties. The objectives of this study were conducted to identify the Tuba plants and quantify their rotenone contents. **Materials and Methods:** Nine tuba plants of different local names were collected from various locations in Peninsular Malaysia. Random Amplification of Polymorphic DNA (RAPD) and internal transcribed spacer (ITS) marker of 9 Tuba plant accessions were used to identify the species. **Results:** Both methods were equally adequate for Tuba plants species identification. Four different species were obtained from nine accessions and they were *Derris elliptica*, *Paraderris elliptica*, *Fordia splendidissima* and *Paraderris piscatoria*. These species are the new record in Peninsular Malaysia. All accessions contained rotenone with the concentration varying from 0.25-1.02 μg mL^{-1}. The highest rotenone content, 1.02 μg mL^{-1} was from *D. elliptica* (Tuba merah). This indicated that the two techniques (RAPD and ITS) are equally appropriate for the analysis of genetic diversity in Tuba plants. **Conclusion:** The study will provide information for the conservation of Tuba plants and further improvement of rotenone contents.

Key words: Tuba plants, rotenone, identification, species, genetic

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Corresponding Author: Norhayu Asib, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia Tel: +603-89474952

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tuba plants known as *Derris* species in Malaysia, Brunei and Indonesia, while other names such as Lai Nam in Thailand, Taubi in Philippines, are commonly found in South East Asia. They are used as fish poison as well as an insecticide[1]. The plant species belong to the Fabaceae family and are perennial shrubs and woody climbers. There are many tuba plants in Malaysia known by various local names such as Tuba Bonsai, Tuba Tualang, Tuba Nerak, Tuba Susu and Tuba Akar Bukit Payung. Identification of plant species can be through out phylogenetic analysis such as the Random Amplified Polymorphic DNA (RAPD) and Internal Transcribes Spacer (ITS) regions. RAPD markers have been used to confirm the existing classification and phylogeny of the rotenone in the Tephrosia. The population and systematic field of study of a variety of different organisms were carry out from analysis of sequence variation in the ITS region[2] and it has potential to differentiate other Tuba species from the region. A study was carried out with the aim of inferring the phylogeny of Tuba species using RAPD marker and ITS markers. There is thus a need to verify the species of tuba plants in Malaysia as a source of rotenone for the quality control of their insecticidal properties. The active component of *Derris* species, rotenone, is mostly obtained from the roots[3]. Rotenone, a naturally occurring compound is an isoflavonoid with acaricidal and insecticidal properties, used to control insect pests of agricultural crops[4]. Rotenone is a fast acting insecticide and rapidly degraded by the sunlight[5]. Rotenone can also be found in other genera of the Fabaceae, such as Tephrosia, Lonchocarpus, Millettia and several species of Mundula[6]. The objectives of this study were to know genetic diversity of Tuba species using molecular marker and to construct the maximum likelihood tree and estimate divergence according to local molecular clock from Tuba species DNA sequences of the internal transcribed spacer region and to identify the name of species from the genus Tuba and to quantify the concentration of rotenone for each species.

MATERIALS AND METHODS

Sampling and DNA extraction: A total of nine Tuba species used in this study were collected from the field at Jerangau, Terengganu (4°57'25''N 103°10'35''E), Tasik Bera, Pahang (3°7'55.7''N 102°36'31''E) and Ladang 10, Universiti Putra Malaysia, Serdang, Selangor (3°1'23''N 101°42'57''E) (Table 1). Young, fresh and with no disease leaves were chosen and extracted for DNA production, as a source for genetic variation among Tuba varieties and species. The DNeasy Plant Mini Kit developed by GeneAll® was used for DNA extraction. The steps for DNA extraction were according to the manufacturer's instruction. Quantification (μg L^{-1}) of amplified DNA after PCR was done utilizing NanoDrop 2000. The DNA material were kept at -20°C for PCR amplification later. This study was conducted for six month including the sampling of Tuba plants at various location. Duration of this study took around one year.

RAPD analysis: Briefly, PCR amplification was carried out a volume of 25 μL. The 24 random primers used in the current study were obtained from Operon Technologies Inc. (Alameda, CA, USA)[7] , these included OPD 1, OPD 2, OPD 3, OPD 4, OPD 5, OPD 6, OPD 7, OPD 8, OPD 9, OPD 10, OPD 11, OPD 12, OPD 13, OPD 14, OPD 15, OPS 3, OPS 5, OPS 7, OPS 8, OPS 12,OPS 14, OPS 16, OPS 17 and OPS 19. Amplification reactions were carried out using the following cycle profile: Initial denaturation at 94°C for 5 min, depends on primer temperature for 1 min, 72°C for 2 min followed by 40 cycles and final at 72°C for 7 min. PCR products were electrophoresed on a 1.0% agarose gel, stained ethidium bromide and viewed using Gel Documentation System (UVP, UK).

Internal transcribed spacer regions (ITS) analysis: Amplifications were carried out 50 μL containing 6.5 μL dNTP mix, 8.5 μL HQ buffer, 1.0 μL *Taq* DNA polymerase, 8.7 μL 10x *Taq* reaction buffer (GeneAll), 3.5 μL DNA template, 3.5 μL

Table 1: List of accessions with location sampling

Samples	Location	GPS coordinate
Tuba Bonsai (TB)	Tasik Bera, Pahang	3°7'55.7''N 102°36'31''E
Tuba Tualang (TT)	Tasik Bera, Pahang	3°7'55.7''N 102°36'31''E
Tuba Merah (TM)	Jerangau, Terengganu	4°57'25''N 103°10'35''E
Tuba Nerak (TN)	Jerangau, Terengganu	4°57'25''N 103°10'35''E
Tuba Susu (TS)	Jerangau, Terengganu	4°57'25''N 103°10'35''E
Tuba Putih (TP)	Jerangau, Terengganu	4°57'25''N 103°10'35''E
Tuba Akar Bukit Payung (TABP)	Jerangau, Terengganu	4°57'25''N 103°10'35''E
Tuba Tuba (TD)	Ladang 10, Universiti Putra Malaysia	3°1' 23''N 101°42'57''E
Tuba Akar (TA)	Ladang 10, Universiti Putra Malaysia	3°1' 23''N 101°42'57''E

forward and 3.5 µL reverse primers and 15.5 µL deionized water. Amplification reactions were carried out using the following cycle profile: Initial denaturation at 94°C for 3 min, 58°C for 1 min, 72°C for 1 min followed by 35 cycles and final at 72°C for 4 min. Sequence reactions were carried out in both directions for each purified double-stranded PCR product using Applied Biosystems Bid Dye Terminator Ver.1.1, buffer and primers.

Data analysis: DNA banding patterns generated by RAPD were scored for the absence (0) or presence (1) of each amplified band. Cluster analysis was based on similarity matrices using the unweighted pair group method analysis (UPGMA) program in NTSYS 2.10e software. Analysis of sequence was done on the ABI Prism 310 and sequences were observed and edited on Chromas Lite 2.0. The ITS region for all accession were determined by comparing the sequences in BLAST (http://www.blast.com). Phylogenetic relationship analysis was led utilizing Mega[8] form 5.

Extraction and quantification of rotenone from Tuba species: The rotenone standard was obtained from Merck Co. All solvents for analysis were HPLC analytical grade and purchased from Sigma Aldrich. The distilled and HPLC grade water were obtained from water purification system (ELGA, USA). The quantification of rotenone was conducted in the Toxicology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia. The roots were cleaned of any fine dirt and soil and dried in an oven overnight at of 28-30°C. The normal soaking extraction method was used to extract the rotenone. The dried roots of 0.5-2.0 mm in diameter (100 g) were placed in a beaker and soaked with 900 mL of acetone at room temperature (28-30°C) and placed in the dark overnight. The liquid crude extracts were filtered through 15 cm Whatman filter paper (number 4) directly into 1 L Erlenmeyer flask after 24 h of extraction[9]. The solvent was evaporated in a rotary evaporator (100 rpm) under vacuum at 40°C for 50 min. The crude extract was then eluted using 10 mL of acetone and stored in a dark container to protect it from light and kept in a refrigerator at 4°C. The yield of extraction was calculated as the percentage of crude extract obtained from the weight (g) of dried roots.

The yield of crude extract was calculated as follows:

$$\text{Yield of extraction } (\%) = \frac{\text{Weight of crude extract}}{\text{Weight of dried roots}} \times 100$$

The filtered liquid crude extracts were transferred into small vials ready for the HPLC analysis of rotenone content with respect to different locations of collected samples. The analytical high performance liquid chromatography instrument consisted of a Water 7996 (USA) photodiode array detector with programmable binary solvent delivery system controller (Water 600), autosampler (Water 717) and Water C18 column (10 µm 3.9×150 mm). The signal was acquired and processed by computer (Compaq) equipped with Empower Pro Software. The controller was connected to the mobile phase, which consisted of a mixture of methanol:water at a ratio of 8:2 (v/v) and with a flow rate of 1.0 mL min^{-1}. The solution was degassed in an ultrasonic bath and filtered under vacuum through a membrane (Milipore, PVDF). The isocratic programme consisted of 100% mobile phase.

RESULTS AND DISCUSSION

Polymorphism RAPD analysis: Nine species were studied for RAPD marker polymorphism. About 24 primers for RAPD marker polymorphism were used as a part of responses with the Tuba plants genomic template and out of which 15 primers were chosen (Table 2). A total of 323 fragments were produced from these primers. The interval range band from 100-1517 base pairs through pairwise genetic distances. From these result, the quantity of polymorphics loci, the percentage of polymorphics loci, Shannon's Information list (I) and the effective number of alleles (Ne) were figured to be 318, 98.45, 0.4571 and 1.4533%, respectively. A dendrogram based of UPGMA analysis of the RAPD information is shown in Fig. 1. UPGMA clustering of RAPD data produced a dendrogram. The genetic distances between accessions ranged from 0.2642-0.7678. All species were assembled as three major clusters with a threshold genetic distance of 0.03. Cluster I comprises of species: Tuba Bonsai (1), Tuba Derris (2), Tuba Tualang (3), Tuba Akar (4), Tuba Nerak (5) and Tuba Merah (6).

Table 2: Size of DNA bands generated by individual RAPD primers per population and characteristics of RAPD primers used for Tuba plant

Name of primers	Sequences of primer	Size of DNA bands (bp)
OPD 3	5' GTC GCC GTC A 3'	100-800
OPD 6	5' ACC TGA ACG G 3'	700-1200
OPD 7	5' TTG GCA CGG G 3'	100-900
OPD 8	5' GTG TGC CCC A 3'	450-1200
OPD 9	5' CTC TGG AGA C 3'	200-850
OPD 10	5' GGT CTA CAC C 3'	100-850
OPD 11	5' AGC GCC ATT G 3'	100-900
OPD 12	5' CAC CGT ATC C 3'	100-850
OPS 3	5' CAG AGG TCC C 3'	100-1200
OPS 5	5' TTT GGG GCC T 3'	150-1000
OPS 7	5' TCC GAT GCT G 3'	200-1517
OPS 8	5' TTC AGG GTG G 3'	200-1517
OPS 14	5' AAA GGG GTC C 3'	600-1517
OPS 16	5' AGG GGG CCA C 3'	100-800
OPS 19	5' GAG TCA GCA G 3'	100-250

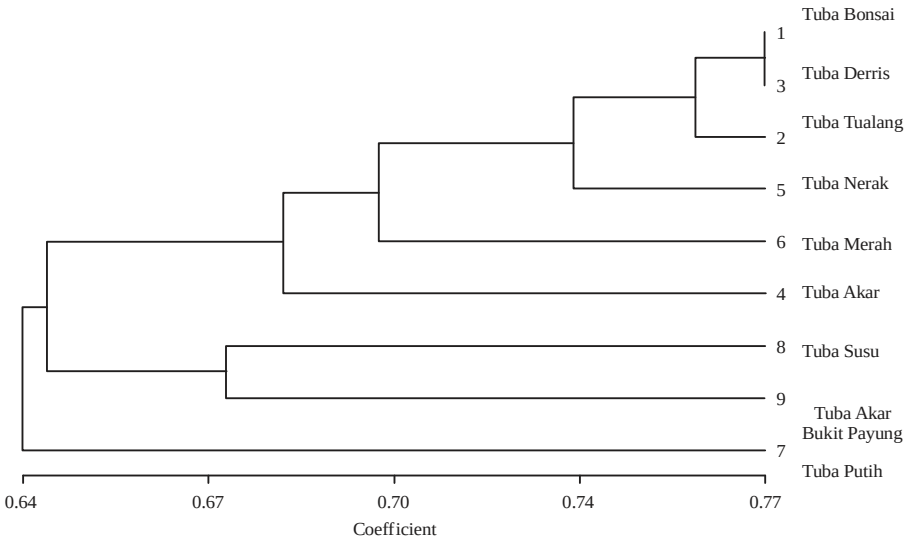

Fig. 1: Dendrogram of genetic relationships among 9 populations of Tuba spp. based on 15 RAPD primers using the UPGMA algorithm and the genetic distances

Table 3: Results from BLAST enquiry matching to the small subunit ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence and large subunit ribosomal RNA gene, partial sequence of three species

Accessions	Seq length (bp)	Match to species	Accession number	Status in Malaysia
Tuba Bonsai (TB)	747	*Paraderris piscatoria*	KJ579427	New record
Tuba Tualang (TT)	734	*Paraderris lionoides*	KJ579433	New record
Tuba Merah (TM)	709	*Derris elliptica*	KJ579429	New record
Tuba Nerak (TN)	736	*Derris elliptica*	KJ579430	New record
Tuba Susu (TS)	694	*Derris elliptica*	KJ579432	New record
Tuba Putih (TP)	740	*Derris elliptica*	KJ579431	New record
Tuba Akar Bukit Payung (TABP)	797	*Fordia splendidissima*	KJ579426	New record
Tuba Tuba (TD)	743	*Derris elliptica*	KJ579428	New record
Tuba Akar (TA)	730	*Derris elliptica*	KJ570975	New record

Cluster II of Tuba Susu (8) and Tuba Akar Bukit Payung (9) and Cluster III of Tuba Putih (7). The correlation coefficient was 0.77 and reproducibility of the RAPD technique averaged 77%. RAPD analysis has been a useful tool for detecting the genetic characterization of nine species of Tuba and broadly used in investigations to reveal the level of genetic diversity among species[10] . Despite the fact that the two Tuba plants Tuba Derris (*D. elliptica*) and Tuba Akar Bukit Payung (*Fordia splendidissima*) were considered as two different species, they are exceptionally comparative in the genetic diversity from the RAPD analysis.

Internal transcribed spacer regions (ITS): This study was revealed the species identity of the nine Tuba species used as a part of this study. The initial step was to compare with the sequences from the Tuba plants to accessible DNA databases in Genbank utilizing the BLAST enquiry stage (Table 3). The result were about 98-99% match to *D. elliptica*, 95% match to *Paraderris elliptica* and 95% to *F. splendidissima* in which the *D. elliptica* and *Paraderris elliptica* were from Thailand. From this interaction, the species plants collected were from the same family and had highly similar to rotenone-containing as an active ingredient. Figure 1 showed that result from aligned ITS sequences from gene bank through phylogenetic analysis. The quantity of nucleotide substitutions per site was under 10 between *D. elliptica*, *Paraderris elliptica* and *Paraderris lianoides* with a 96% bootstrap value. The DNA arrangement of Tuba (Table 3) had a 95% match with *Paraderris elliptica*. On the other hand, one of Tuba accession which is Tuba akar bukit payung had a 95% match to *F. splendidissima*. The DNA sequences of the ITS regions for the nine Tuba plants including the six groups were then adjusted to Muscle bringing about 783 sites with gaps and 621 sites without gaps. There were 378 conserved sites, 385 variable sites and 267 parsimonious and informative sites. The quantity of locales used to produce the phylogenetic tree was 457. The maximum likelihood tree in Fig. 2 demonstrated that the nine Tuba plants were isolated from the group and

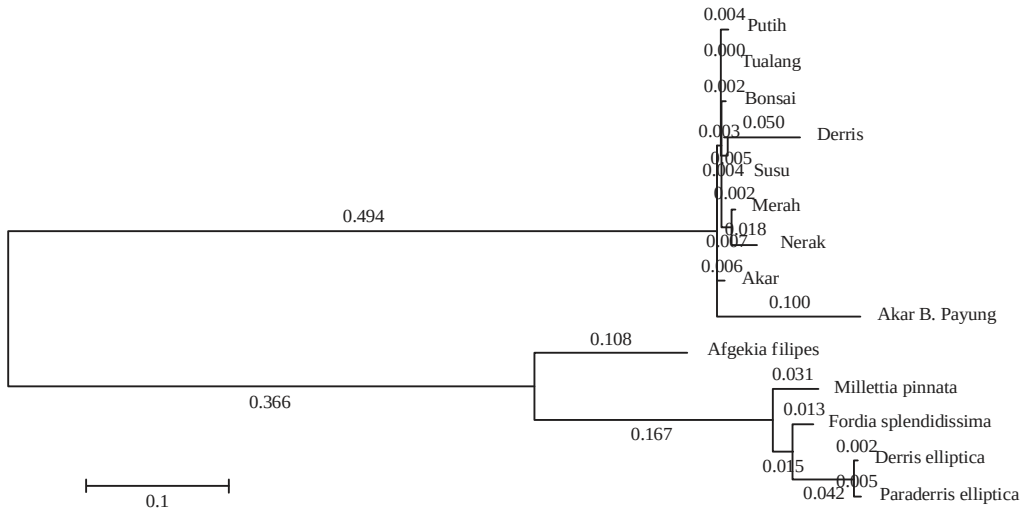

Fig. 2: Maximum likelihood tree replicated 1000 times with highest bootstrap value recorded at 78% and number of substitution per site is below the branch across 602 parsimonious and informative sites

Table 4: Distance and number of nucleotide substitutions between each species that has less than 10 number of nucleotide substitutions

Species 1	Species 2	Distance	Number of nucleotide substitution
Akar	Bonsai	0.0111	5
Akar	Merah	0.01801	8
Bonsai	Merah	0.01114	5
Merah	Nerak	0.02026	9
Akar	Putih	0.01337	6
Bonsai	Putih	0.00662	3
Merah	Putih	0.01343	6
Akar	Susu	0.01339	6
Bonsai	Susu	0.0111	5
Merah	Susu	0.01801	8
Putih	Susu	0.01337	6
Akar	Tualang	0.00886	4
Bonsai	Tualang	0.0022	1
Merah	Tualang	0.00891	4
Putih	Tualang	0.0044	2
Susu	Tualang	0.00886	4
Derris elliptica	*Paraderris elliptica*	0.00665	3

were characterized with *D. elliptica* and *Paraderris elliptica* despite the fact that they coordinated more than 95% in the BLAST analysis. According to the ITS phylogeny in this study, the Tuba Akar Bukit Payung was not in the *Derris* genus group. It belongs to *Fordia* genus, which has different leaf characteristics and also matched to *Fordia* genus compared with genebank[11]. However, Tuba Derris matched to *Paraderris piscatoria* and Tuba Tualang matched to *Paraderris lionoides* compared to genebank. These species were found in Asia Tropics, Philippines[12]. Paraderris has to be synonymized with *Derris*[13]. Table 4 showed that their number of nucleotide substitutions refer to the Tuba plants with the exception of Tuba Derris (*D. elliptica*) and Tuba Akar Bukit Payung (*Fordia splendidissima*) had a distinction of under 10 nucleotide substitutions with one another, showing that these 7 Tuba plants are of the same species. With respect to Tuba Derris (*D. elliptica*) and Tuba Akar Bukit Payung (*Fordia splendidissima*), they are of diverse species. None of the Tuba plants are the same species as the group. The best ITS barcode should be relatively short in length (~700 bp), more variable between than within species and easily amplifiable with universal primers[14]. The reproductive system determines the way genes are transmitted between generations and consequently, the levels of genetic variability are closely related to this[15]. The independence of the molecular clock from adaptive evolution deserves further investigation[16].

Extraction and quantification of Tuba species: Table 5 showed the yield of extracts and concentrations of rotenone from normal soaking extraction process of Tuba plants collected from various locations in Peninsular Malaysia. Tuba Merah (*D. elliptica*) collected from Jerangau, Terengganu gave the highest yield followed by Tuba Derris (*D. elliptica*) from Universiti Putra Malaysia, Serdang. Furthermore, Tuba species has contained rotenone which is one of the source active ingredients for bio-pesticide to control insect pest and not to harmful to human and environment[9]. The different concentration of rotenone contained in the Tuba species because of their own habitat and handling extraction method of the root size[9].

Table 5: Different concentrations of rotenone extracted from various Tuba spp.

Samples	Scientific name	Sample collection	Rotenone concentration (μg mL^{-1})
Tuba Bonsai	*Paraderris piscatoria*	Tasik Bera, Pahang	0.75^{d}
Tuba Tualang	*Paraderris lianoides*	Tasik Bera, Pahang	0.87^{c}
Tuba Merah	*Derris elliptica*	Jerangau, Terengganu	1.02^{a}
Tuba Nerak	*Derris elliptica*	Jerangau, Terengganu	0.25^{h}
Tuba Susu	*Derris elliptica*	Jerangau, Terengganu	0.32^{g}
Tuba Putih	*Derris elliptica*	Jerangau, Terengganu	0.44^{f}
Tuba Akar Bukit Payung	*Fordia splendidissima*	Jerangau, Terengganu	0.39^{g}
Tuba Tuba	*Derris elliptica*	UPM, Serdang	0.94^{b}
Tuba akar	*Derris elliptica*	UPM, Serdang	0.57^{e}

*Different letter showed the significant different at $p<0.05$

CONCLUSION

The potential technique using RAPD-molecular markers were assessed for the genetic characterization of 9 species of Tuba UPGMA statistical methods to analyze the data presented their power to reveal genetic relationship. The maximum likelihood tree and divergence according to local molecular clock from Tuba species shows the DNA sequences of the internal transcribed spacer region were constructed and name of species from the genus *Derris* were identified as a new record in Malaysia. Out of 9 species collected, only 4 species are different species which are TB (KJ579427), TM (KJ579429), TN (KJ579430), TS (KJ579432), TP (KJ579431) and TA (KJ570975) were identified as *Derris elliptica*, TT (KJ579433) was identified as *Paraderris lionoides*, TABP (KJ579426) was identified as *Fordia splendidissima* and TD (KJ579428) was identified as *Paraderris piscatoria*. The active ingredient (a.i.), rotenone that was extracted from the roots of Tuba plants using acetone contained 1% of the rotenone. Therefore, every species of Tuba plants showed different of quantity of rotenone content. This study showed the importance of genetic identification to identify the real species of Tuba plants and rotenone content for each species.

SIGNIFICANCE STATEMENT

This study revealed the potential distribution of Tuba plants species that can be beneficial for the local communities as a bio-pesticide against insect pest in vegetables. These findings will help researchers to reveal the areas of Tuba plants distribution and spread information to others who are eager for it. Thus new information distribution and active ingredient content on Tuba plants can be obtained.

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