



## Genetic Diversity of *Gluta* lacquer Clones in Northeastern Thailand Using by Start Codon Targeted (SCoT) markers

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### Abstract

**Background and Objective:** Lacquer is a special natural product in Southeastern Asia and Eastern Asia that was considered to be coating materials used in mankind culture for thousand years. In Asian countries, lacquer sap is known and obtained from 5 species; *Toxicodendron vernicifluum* in China, Japan and Korea, *T. succedaneum* in Vietnam and Taiwan, *G. usitata* in Myanmar and Thailand, *G. laccifera* in Thailand and Cambodia and *G. glabra* in Thailand. Eleven *Gluta* species are natural distribution Thailand. As of today, the lacquer tree habitats have been loss by anthropogenic activities. Mostly, the lacquer trees have remained in protected areas, while in land with a holding has been effective by changing of land ownership. The objective of this study was to estimate genetic diversity among species & clones in northeastern Thailand using SCoT markers.

**Methodology:** A total of 3 *Gluta* species were selected for the present study consisting of 1 clone of *Gluta cambodiana*, 13 clones of *G. laccifera* and 57 clones of *G. glabra*. Plant DNA extraction was isolated from the leaf tissues according to the CTAB method. Forty-five SCoT primers screened, using 1 clone of each species, for polymorphism and reproducibility. Each 20  $\mu$ l amplification reaction consisted of 2  $\mu$ l X 10X polymerase chain reaction (PCR) buffer (with 25 mM MgCl<sub>2</sub>), 0.4  $\mu$ l dNTP (10 mM), 1  $\mu$ l of each primer (10  $\mu$ M), 0.2  $\mu$ l My Tag™ DNA polymerase, 15.4  $\mu$ l distilled water, and 1  $\mu$ l cDNA template (20 ng) [1]. My Tag™ DNA polymerase and buffer were purchased from Bio Line (Bioline Regents Ltd, USA). The procedure for the PCR reaction using a thermo cycler C1000 (Bio-Rad, USA) include an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 50 s, 50°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 5 min. The PCR amplifications for all primers were processed using the same procedure, and all amplified products were resolved on 1.5% agarose electrophoresis in 1% Tris-Borate/ EDTA buffer and stained with ethidium bromide (0.5  $\mu$ g/ml). The banding pattern images were acquired under UV light using a SynGene Gel-Doc Imaging System (SYNGENE, USA). Genetic similarity among clone was evaluated by calculating the Jaccard's similarity coefficient and cluster analysis was performed using the UPGMA using NTSYS-PC software package.

**Result:** Of the 45 SCoT primers tested in the present study, 10 primers were selected for genetic relationship and diversity analysis with allele size ranging from a maximum of 3,000 bp to a minimum of 400 bp. Among the 71 clones, 10 SCoT primers generated 53 bands with an average of 5.30 bands per primer ranging from 4 to 8 per primer, of which 37 were polymorphic, yielding a polymorphism rate of 69%. These results indicated that a high polymorphism could be detected among *Gluta* clones using SCoT markers. Genetic similarity coefficient between pairs of accessions was obtained from the markers data based on Jaccard's similarity coefficient. Pairwise comparison of accessions indicated relative genetic similarity between clone ranging from a maximum of 0.97 to a minimum of 0.36 with an average of 0.67. The similarity coefficients generated from the SCoT data were used to construct a dendrogram based on the simple matching Jaccard's similarity coefficient. In the present studies, the dendrogram grouped the 71 clones into 5 clusters at a similarity index of 0.61.

**Conclusion:** The study on genetic diversity of 71 selected *Gluta* lacquer clones from northeastern Thailand analyzed using by 10 suitable SCoT markers. The result showed polymorphisms in ranges 400 - 3,000 bp, total 57 bands, average 5.7 bands per primer and 37 polymorphic bands or 69%. Jaccard's similarity coefficient was ranged from 0.36 - 0.97. A dendrogram constructed using unweighted pair group method with arithmetic mean (UPGMA) of *Gluta* lacquer clones classified into 5 clusters at 0.61 of genetic similarity coefficient.

**Keywords:** Genetic Diversity; *Gluta* Species; Lacquer; Northeastern Thailand; Scot Primer

## Introduction

Lacquer is a special natural product in Southeastern Asia and Eastern Asia that was considered to be coating materials used in mankind culture for thousand years. In Asian countries, lacquer sap is widely obtained from several species; Chinese lacquer (*Toxicodendron vernicifluum* (Stokes) F. Barkley) in China, Japan and Korea, wax tree (*T. succedaneum* (L.) Kuntze) in Vietnam and Taiwan, Burmese lacquer (*G. usitata*) in Myanmar and Thailand [2] and Laccifera lacquer (*G. laccifera*) in Thailand and Cambodia [3, 4]. Lacquer sap is a raw material to use for many lacquer works, such like; mother-of-pear inlaid, colored glass inlay, traditional Thai painting (Lai Rot Nam, Lai Kammalor), varnish for headgears (Khon mask, crown, tiara, etc.), military armors, etc. [5,6]. Historically, the oldest archaeological evidence of lacquer utilization in Thailand was found on log coffins at Pang Mapha district, Mae Hong Son Province which could be dating with range  $1,960 \pm 30$  years to  $1,636 \pm 44$  years [7]. During AD 1864-1865 (early Bangkok period), lacquer sap was recorded for annual gift tribute account on lacquer sap that was submitted from Meaung Udongmeechai (Cambodia) to Siam [8] that indicated lacquer sap very important for lacquer ware handicraft. In 1959, Thailand had recorded to export lacquer exuded to Japan an amount ex exceeded 100 -150 tons [9]. The average volume of lacquer sap used in the country before 1989 at 8 tons [10]. In 2015, the lacquer sap production in Thailand was estimated less than 500 kg/year. However, it has decrease continuously after Thailand logging ban in 1989, and has import from neighboring countries now. Because from the past to present, lacquer sap was tapped from natural forest and did not establish the lacquer tree and no promotion to industry scale. As of today, the lacquer tree habitats have been loss by anthropogenic activities. Mostly, the lacquer trees have remained in protected areas, while in land with a holding has been effective by changing of land ownership [10].

The species diversity of the genus *Gluta* in the world has about 30 species [11] while has recorded 34 accepted species in [12], its species number has recorded 11 species in Thailand, namely; *Gluta cambodiana* Pierre, *G. compacta* Evrad, *G. elegans* (Wall.) Hook.f., *G. glabra* (Wall.) Ding Hou, *G. laccifera* (Pierre) Ding Hou, *G. obovata* Craib, *G. renghas* L., *G. tavoyana* Wall. ex Hook.f., *G. usitata* (Wall.) Ding Hou, *G. velutina* Blume and *G. wrayi* King [13]. *Gluta glabra* is widely distribution more than others species, mainly in northeastern part, while *Gluta usitata* is very important for lacquer ware industry, which has limited in northern part of Thailand up to Myanmar. *Gluta laccifera* is scattered distributed in some areas of northeastern and upper

peninsula Thailand extending to northern Cambodia [10]. The other *Gluta* species are a little population in the natural forests with conservation status rare species.

Molecular markers have proven to be invaluable tools for assessing plants' genetic resources by improving our understanding with regards to the distribution and the extent of genetic variation within and among species. Recently developed marker technologies allow the uncovering of the extent of the genetic variation in an unprecedented way through increased coverage of the genome [14]. [15] reported a simple and novel DNA marker technique Start Codon Targeted (SCoT) polymorphism. It uses 18-mer single primer in PCR and PCR products are resolved using standard agarose gel electrophoresis. The primers are very easy to design based on the conserved region surrounding the translation initiation codon, ATG [16, 17] without needing the genomic sequence information. The SCoT marker system is useful for identification and genetic diversity and variation analysis in agricultural and forestry plant species such like; mango [1, 18, 19], wax tree (*Rhus succedanea* L.) [20] grape [21], orchid genus *Eria* [22], *Dendrobium nobile* [23] and *Diospyros* species [24], etc. The objective of this study was to estimate genetic diversity among species and clones in northeastern Thailand using SCoT markers. Rich genetic diversity within and among population thus provides an important basis for maintaining their wisdom and enabling sustainable lacquer sap tapping. In view of the importance of lacquer tree in Thailand and Worldwide, this study aimed at characterizing the genetic diversity of 71 *Gluta* clones of northeastern Thailand using SCoT markers polymorphism, and to evaluate the usefulness of SCoT markers for germplasm management.

## Materials and Methods

### Plant Material

A total of 3 *Gluta* species were selected for the present study consisting of 1 clone of *Gluta cambodiana*, 13 clones of *G. laccifera* and 57 clones of *G. glabra* (Table 1). The experimental set consisted of 71 samples of *Gluta* species from three biogeographically areas in northeastern Thailand ; upper northeastern (UNT) consisting of 19 clones from Udon Thani (A6, A29 – A44, A60, A61) 2 clones from Sakorn Nakhon (A3, A4), 6 clones from Kalasin (A2, A5, A45, A46, A47, A48), 7 clones from Khon Khaen (A62, A63, A64, A65, A66, A67, A68), western northeastern (WNT) consisting of 23 clones from Chaiyaphum (A1, A7 –A28) and lower northeastern (LNT) consisting of 11 clones from Nakhon Ratchasima (A49 –A59) and 3 clones from Ubol Ratchathani (A69, A70, B1) (Table 1). Young and healthy leaf samples of *Gluta* species were collected from natural forests in 3 biogeographically areas in northeastern Thailand.

Species	Sample ID	Collection sample sites in NE Thailand		Total
		Geographic origin <sup>1)</sup>	Collection site	
<i>Gluta cambodiana</i>	B1	LNT	Ubon Ratchathani	1
<i>Gluta glabra</i>	A1, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A21, A22, A23, A24, A25, A26, A27, A28	WNT	Chaiphaphum	23
	A2, A5, A45, A46, A47, A48	UNT	Kalasin	6
	A3, A4,	UNT	Sakorn Nakhon	2
	A6, A29, A30, A31, A32, A33, A34, A35, A36, A37, A38, A39, A40, A41, A42, A43, A44, A60, A61	UNT	Udon Thani	19
	A62, A63, A64, A65, A66, A67, A68	UNT	Khonkhaen	7
<i>Gluta laccifera</i>	A49, A50, A51, A52, A53, A54, A55, A56, A57, A58, A59	LNT	Nakhon Ratchasima	11
	A69, A70	LNT	Ubon Ratchathani	2

Remark: Geographic origin<sup>1)</sup> = LNT; Lower northeastern Thailand, WNT; Western northeastern Thailand, UNT; Upper northeastern Thailand.

**Table 1:** List of the *Gluta* samples in this study.

## DNA Extraction

From each sample, 0.5 g of young and healthy leaf tissue was collected and was either immediately used for DNA extraction, or stored in silica gel until completely dry before DNA isolation. Genomic DNA was isolated from the leaf tissues according to the CTAB method described by [25] with minor modifications [26]. DNA samples were stored at -20°C and the quality verified by electrophoresis on ethidium bromide stained 1% agarose gel.

## SCoT Analysis

All SCoT primers were synthesized by Bio Design Co Ltd. (Pathumthani, Thailand). Forty-five SCoT primers were initially screened, using 1 clone of each species, for polymorphism and reproducibility. Each 20 µl amplification reaction consisted of 2 µl X 10X polymerase chain reaction (PCR) buffer (with 25 mM MgCl<sub>2</sub>), 0.4 µl dNTP (10 mM), 1 µl of each primer (10 µM), 0.2 µl My Tag<sup>TM</sup> DNA polymerase, 15.4 µl distilled water, and 1 µl cDNA template (20 ng) (Luo et al., 2010). My Tag<sup>TM</sup> DNA polymerase and buffer were purchased from Bio Line (Bioline Regents Ltd, USA). The procedure for the PCR reaction using a thermo cycler C1000 (Bio-Rad, USA) include an initial denaturation step at 94°C for 3 min, followed by 36 cycles of 94°C for 50 s, 50°C for

1 min, 72°C for 2 min, and a final extension at 72°C for 5 min. The PCR amplifications for all primers were processed using the same procedure, and all amplified products were resolved on 1.5% agarose electrophoresis in 1% Tris-Borate/ EDTA buffer and stained with ethidium bromide (0.5 µg/ml). The banding pattern images were acquired under UV light using a SynGene Gel-Doc Imaging System (SYNGENE, USA).

## Data Analysis

The band patterns obtained by each SCoT primer were scored as absent (0) or present (1). Only clear, reproducible bands were scored. Genetic similarity among clone was evaluated by calculating the Jaccard's similarity coefficient and cluster analysis was performed using the UPGMA (Unweighted Pair Group Method of Arithmetic Means) Algorithm using NTSYS-PC software package [27]. A dendrogram was produced from the Jaccard's similarity coefficient matrices for each marker type to investigate relationships among genotypes.

## Results and Discussion

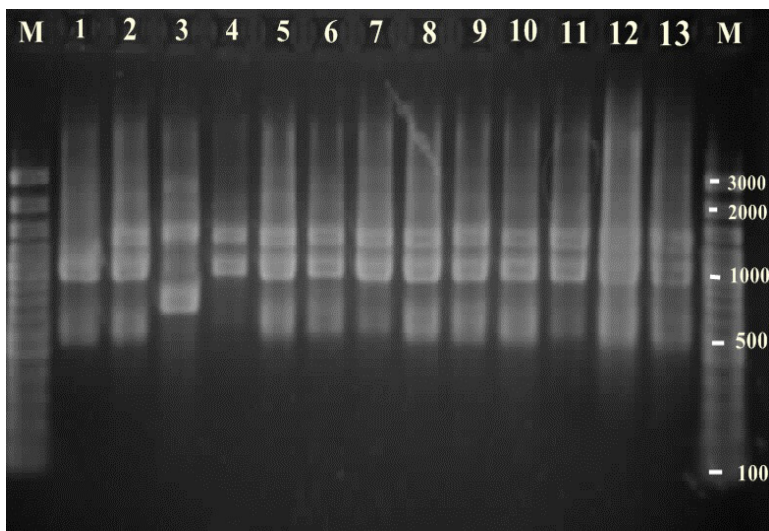
### Polymorphism Detected Using SCoT Markers

The SCoT primers were screened according to [1]. Of the 45

primers (Primer ID 37- 80) tested in the present study, 10 primers were selected for genetic relationship and diversity analysis with allele size ranging from a maximum of 3,000 bp (SCoT43 and SCoT48) to a minimum of 400 bp (SCoT47 and SCoT55). Among the 71 clones, 10 SCoT primers generated 53 bands with an average of 5.30 bands per primer ranging from 4 (SCoT54, SCoT58, SCoT63, SCoT67) to 8 (SCoT43) per primer, of which 37 were polymorphic, yielding a polymorphism rate of 69% (Table 2). The number of polymorphic bands varied from 2 (SCoT61 and SCoT67) to 6 (SCoT43 and SCoT55) (Figure 1), with an average of 3.7 bands per primer (Table 2). The primer SCoT55 amplified the highest percentage of polymorphic bands (86%) (Table 2). No single SCoT primer could distinguish all the clones independently. These results indicate that SCoT markers could detect high polymorphism among *Gluta* clones. These results indicated that a high polymorphism could be detected among *Gluta* clones using SCoT markers. All the primers which used in this study have been performed three times on different days and using different thermal cycles, no new bands were detected.

Primer ID	Sequences (5'-3')	Annealing temperature (°C)	% GC	Band size (bp)	Total bands number	Polymorphic bands number	%Polymorphic bands
SCoT38	CAATGGCTACCACTAACG	50	56	500 -1,500	5	4	80
SCoT43	CAATGGCTACCACCGCAG	61	61	700 -3,000	8	6	75
SCoT47	ACAATGGCTACCACTGCC	56	56	400 -2,000	6	4	67
SCoT48	ACAATGGCTACCACTGGC	56	56	700 -3,000	6	4	67
SCoT54	ACAATGGCTACCACCGC	56	56	500 -1,500	4	3	75
SCoT55	ACAATGGCTACCACTACC	50	50	400 -1,500	7	6	86
SCoT58	ACAATGGCTACCACTAGG	50	50	700 -1,500	4	3	75
SCoT61	CAACAATGGCTACCACCG	56	56	700 -2,000	5	2	40
SCoT63	ACCATGGCTACCACGGGC	67	67	600 -1,500	4	3	75
SCoT67	ACCATGGCTACCAGCGGC	67	67	600 -1,500	4	2	50
<b>Total</b>	-	-	-	-	53	37	69
<b>Average</b>	-	-	-	-	5.3	3.7	6.9

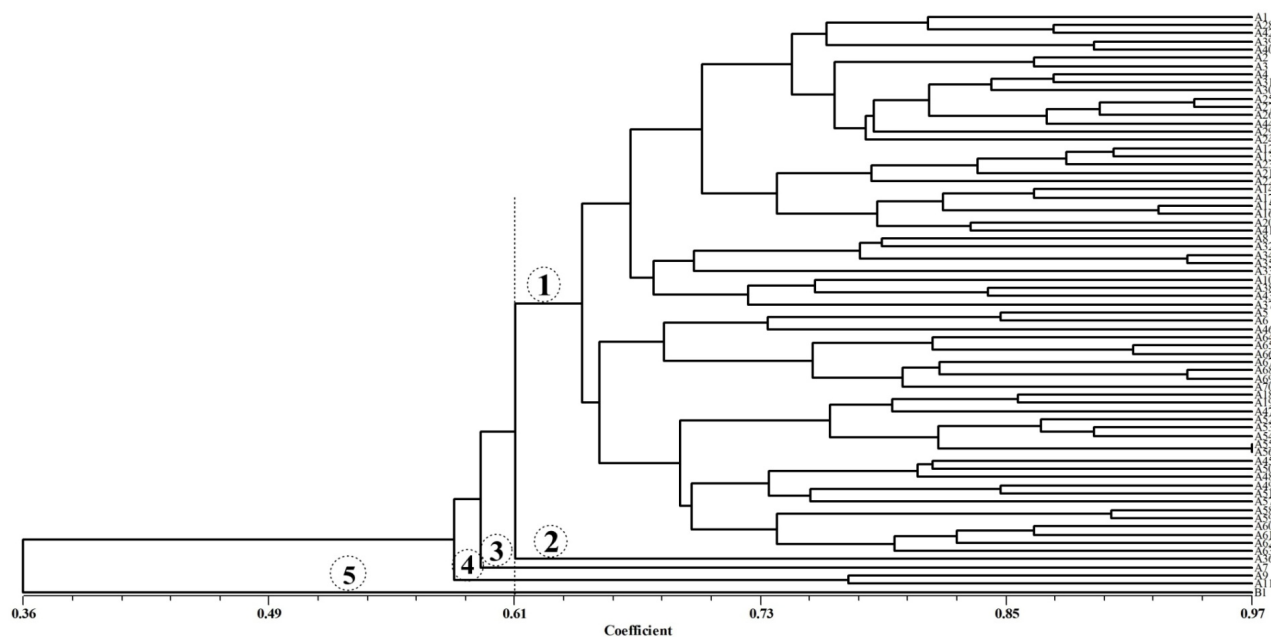
**Table 2:** List and sequences of each primer and their total bands number, polymorphic band number, polymorphic band percentage of 71 samples of *Gluta* samples as revealed by SCoT markers.



**Figure 1:** Application products generated by SCoT 63 with 13 clones of *Gluta* species. Lane M shows the 100 bp DNA ladder plus and Lane 1-13; A21, A25, A24, A39, A22, A23, A42, A26, A27, A28, A29, A30, A31, respectively.

### Genetic Diversity Analysis Among the Tested *Gluta* Clones

Genetic diversity revealed by SCoT markers All the 53 scored bands were used to calculate genetic similarity among the 1 clone of *G. cambodiana*, 13 clones of *G. laccifera* and 57 clones of *G. glabra*. Genetic similarity coefficient between pairs of accessions was obtained from the markers data based on Jaccard's similarity coefficient. Pairwise comparison of accessions indicated relative genetic similarity between clone ranging from a maximum of 0.97 to a minimum of 0.36 with an average of 0.67(Figure 2). The observed highest genetic similarity value (0.97) was found in clone ID No. A55 & A56 of *G. laccifera*, the observed lowest genetic similarity value (0.36) was found in clone ID No. B1 of *G. cambodiana*. The clones of *G. glabra* showed the widest of the genetic similarity 0.57 and 0.953, as a result of the highest of sample number (Figure 2).



**Figure 2:** Dendrogram of the 71 *Gluta* samples studied based on UPGMA analysis of 10 SCoT primers using the Jacquard's similarity coefficient.



The similarity coefficients generated from the SCoT data were used to construct a dendrogram based on the simple matching Jaccard's similarity coefficient. In the present studies, the dendrogram grouped the 71 clones into 5 clusters (Figure 2) at a similarity index of 0.61. The cluster '1' consisted of 66 clones, which belonged to 2 species (53 clones of *G. glabra* and 13 clones of *G. laccifera*). This cluster is subdivided into 2 groups at the coefficient 0.637. The first subgroup '1A' consisted of 33 clones of *G. glabra* which originated from upper northeastern and western northeastern Thailand and the second subgroup '1B' consisted of 20 clones of *G. glabra* which originated from upper northeastern Thailand and 13 clones of *G. laccifera* which originated from lower northeastern Thailand. The cluster '2' consisted of only 1 clone of *G. glabra* (A36) which originated from upper northeastern Thailand (Udonthani). The cluster '3' consisted of only 1 clone of *G. glabra* (A7) which originated from western- northeastern Thailand (Chaiyaphum). The cluster '4' consisted of 2 clones of *G. glabra* (A9, A11) which originated from western- northeastern Thailand (Chaiyaphum). The cluster '5' consisted of 1 clone of *Gluta cambodiana* which was classified clearly based on both its fruit morphological characteristics without fruit wing and genotype.

Assessment of genetic diversity among and within plant species is important for breeding and genetic resource conservation programs. To assess the genetic diversity and variation in lacquer species were reported only in the genus *Toxicodendron*, such like, [20] reported in 96 samples of wax tree in Thailand using SCoT primers indicated that was classified into 10 groups with genetic variation was high and [28] reported in 109 cultivars of Chinese lacquer belonging to five different lacquer cultivars (Damu, Dahongpao, Gaobazhi, Huangrongguizhou and Huangmao) in China analyzed by SSR markers indicated that Gaobazhi cultivar had greater genetic diversity and Gaobazhi cultivar was closer to the group containing the Dahongpao and Huangrongguizhou cultivars than Damu and Huangmao cultivars. [29] had attempted to determinate of genetic relationships among *Rhus succedanea* (synonym of *Toxicodendron suucedaneum*) in Japan by 12 microsatellite markers that showed with numbers of alleles per locus ranging from 3 to 19 and expected heterozygosity ranging from 0.22 to 0.85. While [30] combined molecular method of ISSR, AFLP analysis and RAPD markers concerning the superior candidate individuals of *Rhus succedanea* for clone identification and genetic relationship among clone.

[31] reported the genus *Gluta* was closely to mango (genus *Mangifera*), it was classified in the tribe Anacardiaceae, same with the genus *Mangifera*. In this study, it is the first report of the genetic diversity assessment of lacquer species of the genus *Gluta*. The result shows *G. laccifera* that is a sister group of *G. glabra* to be consistent with its flower morphological characteristics having

closely by hairy ovary stalk [32]. By the past, *G. glabra* could provided sap to use a material for lacquer ware handicraft, which was mixed with other sap of lacquer species including *G. laccifera*, its sap was called in "Rak Chaiya", name of its origin from Chaiya district, Surat Thani province where locate in peninsular Thailand. Its sap was super quality, whereof the artisan needed to use a material for lacquer ware making. Both *G. laccifera* and *G. glabra* in under name of Burmese lacquer or 'Rak yai' (*G. usitata*) by Thai craftsmen, whereas three *Gluta* species are closely morphological characters with having 30 stamens, fruit having 5-6 large wings while other *Gluta* species absent wing [13]. The natural gene pool *Gluta* species is poorly represented in the natural habitats. The area of adaptation of *Gluta* species is found often forest fragments which are highly vulnerable to deforestation and urbanization. Thus, conservation of the *Gluta* species gene pool is absolutely essential to revive and maintain genetic variability in natural conditions.

## Conflict of Interest

None.

## Conclusion

This study on genetic diversity of 71 selected *Gluta* lacquer clones from northeastern Thailand analyzed using by SCoT markers. Ten suitable of 45 SCoT primers showed clearly polymorphisms in ranges 400 - 3,000 bp, via; SCoT38, SCoT43, SCoT47, SCoT48, SCoT54, SCoT55, SCoT58, SCoT61, SCoT63 and SCoT67 primers. The result showed total 57 bands, average 5.7 bands per primer and 37 polymorphic bands or 69%. Jaccard's similarity coefficient was ranged from 0.36 - 0.97. A dendrogram constructed using unweighted pair group method with arithmetic mean (UPGMA) of *Gluta* lacquer clones classified into 5 clusters at 0.61 of genetic similarity coefficient.

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