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GENETIC VARIATION OF TEAK MISTLETOE (*Dendrophthoe pentandra* (L.) MIQ.) BASED ON RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS**

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ABSTRACT

Mistletoes are hemiparasitic macroparasite plants which interfere with trees and other wild plants in nutrient acquisition. As the plant has low leaf water potential, it draws water from teak wood tissues during the deciduous stage of the teak host, thereby killing the twigs and eventually, the teak tree. Mistletoes are also a key player in plant diversity. Therefore, the mistletoe population needs to be regulated not only as a parasite but also as a keystone species affecting biodiversity. Knowledge scarcity on the status of mistletoes includes its genetic variation. Hence, the purpose of this study is to analyze the level of genetic variation of teak mistletoe (*Dendrophthoe pentandra*) using RAPD marker. At Padangan teak Clonal Seed Orchard (CSO), it was randomly collected leaf samples from three layers of the mistletoe's crown (upper, middle, and below) were taken from five host teak trees randomly selected from each of the sub-observation measure plots (OMP). Four OMP units inside the observation sample plots (OSP) (n = 3, 50 x 50 m) at different levels of infestation (light, moderate and heavy) were established. Analysis of the genetic variation and genetic distance of mistletoes hanging on the different crown layers were conducted using RAPD markers. The leaf samples from the crown layers, UU (upper crown and sub-section upper), UM (upper crown and sub-section middle), and UB (upper crown and sub-section below), which include U (upper crown) had significantly greater genetic variation (He = 0.181 – 0.255) than those from M layer (middle crown, He = 0.227) and the B layer (below crown, He = 0.114). Furthermore, the widest genetic distance significantly occurred between the mistletoes of the UB and B crown layers (0.310), whereas the nearest genetic distance significantly occurred between mistletoes of UU and UM layers in the upper crown (0.038). Practical implications of the low genetic variation in this study include the control of mistletoe *D. pentandra* infestation by means of restricting its population so that Perhutani State Owned Forestry Enterprise can maintain the level of damage below the economic threshold.

Keywords: *Dendrophthoe pentandra*, genetic variation, mistletoe, RAPD, Teak

INTRODUCTION

Mistletoes are hemiparasitic macroparasite plants which interfere in the nutrient acquisition of cultivated plants including trees and other wild plants. Based on their habitat, mistletoes attach themselves to certain parts of a host plant, such as branches, twigs, and occasionally stems. Many research aspects about mistletoe interaction with their hosts and bird dispersers have been conducted in both plantation and natural forest ecosystems. However, there is a lack of information concerning the effect of environmental variability of the canopy on the fate of mistletoe seeds and seedlings growth (Mellado & Zamora 2014b), include aspect of genetic variation in this study and DNA barcode characterization (Muttaqin *et al.* 2017).

Mistletoe infestations can decrease the production of quality seeds in seed orchards and in timber plantations. Alarmingly, with repeated infestations, mistletoes can kill the host trees.

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The initial process of growth and development of mistletoes takes place when mistletoe seeds spread on those parts of stem by the assistance of birds as main agents (Mellado & Zamora 2014a). The seeds then germinate and develop to form the haustorium organ which penetrates into the xylem of a host (xylem tapping) to absorb important nutrients such as water, minerals, and components of sugar and amino acids.

A study on the ecophysiology of teak and its canopy hemiparasite *Dendrophthoe falcata* var. *pubescens* revealed that mistletoes always maintain lower leaf water potential compared to the host teak. During the deciduous stage of the host teak, the mistletoe is drawing most of the water from the neighbouring wood tissue, thereby causing death of the twigs, and finally the death of the teak. The water use efficiency of the mistletoe is lower compared to that of the teak. Its photosynthetic performance concerning electron transport ability and quantum-energy-use efficiency are better in the mistletoe leaves. Since K and Na are phloem mobile minerals, these are highly concentrated in the misletoe leaves. That is indicative of the absence of any phloem connections between the host and the mistletoe. The mistletoe can photosynthesize at shade and exposed condition, showing its high adaptability to the host (Kallarackal *et al.* 2003). Moreover, mistletoe-host plants' antagonistic interactions, along with bird dispersers, may form complex networks whose function and structure can influence fragmentation at different scales, e.g., molecular or population levels (Arroyo *et al.* 2013).

In the Central Oregon, USA, pruning was effective in controlling the severity of dwarf mistletoes and in increasing the longevity of Douglas-fir, even if not all the misteltoes were removed because of the delayed mistletoe intensification (Maffei *et al.* 2016). In Perhutani, mistletoe control by silvicultural method was also applied by planting 'kersen' (*Muntingia calabura*), together with kesambi plant, dowet (*Syzygium cumini*) and salam (*Syzygium polyanthum*), as edge and filler plants. These plants, serving as substitute hosts of teak, have fruits which are foraged by the primary agent of mistletoe dispersal, the cabai bird (*Dicaeum* sp). The mix planting of teak for mistletoe control was also conducted in Indonesia (Corryanti *et al.* 2012).

Three mistletoe species were found at Padangan teak clonal seed orchard (CSO), East Jawa Province, Indonesia, namely *Dendrophthoe pentandra* (Loranthaceae), *Macrosolen tetragonus* (Loranthaceae), and *Viscum articulatum* (Santalaceae) (Muttaqin *et al.* 2017). *D. pentandra was* the most numerous and the most widely distributed at the orchard. Those mistletoes belong to indigenous plant groups that usually grow on suitable host plants and spread to tropical regions including Indonesia's forested areas.

The main goal of sustainable management at Padangan orchard is to control mistletoe infestation and develop immediate conservation measures. It requires synergistic, not antagonistic, support through the conservation of genetic variation of teak mistletoes which can be a valuable input and be correlated with the result of assessing the intensity of mistletoe infestation, including the True Mistletoe Rating (TMR) modified 8-class rating requirement (Muttaqin 2016). Sustainable management also requires accurate data or information on the level and range of mistletoes genetic variation that validates the presence or scarcity of mistletoe species exhibiting mistletoe main characters and high level of adaptation to environmental changes. The value of high genetic variation would take effect towards species ability to adapt on environmental condition, and vice versa. So as, this study hypothesis has two alternatives; if genetic variation of *D. pentandra* is low then the control of this mistletoe would be restricted to moderate and heavy infestation. If the genetic variation is moderate until high then the control of this mistletoe would be conducted against all levels of infestation from light, to moderate, to heavy. Therefore, knowledge about the genetic variation in mistletoes will improve the sustainable management of teak seed orchards, especially protection from pests and diseases, like mistletoes. Also, it be constitute candidate population for inclusion in future conservation programmes for mistletoe of *Dendrophthoe pentandra* that grow on teak stand in Perhutani area, Indonesia.

For genetic resource conservation, the genetic diversity and population structure of mistletoe *D. pentandra* need immediate investigation (Kim *et al.* 2017). Studies on the

genetic variation of mistletoes had applied some markers such as RAPD, Microsatellite, AFLP (Crichton *et al.* 2012; Yi *et al.* 2013; Amico *et al.* 2014; Kim *et al.* 2017) and the study on the desert mistletoe *Phoradendron californicum* (Santalaceae) had used isolation of 18 Microsatellite loci (Arroyo *et al.* 2013). Despite the ecological and medical importance of *D. pentandra*, only few studies were conducted (Poerba & Sunaryo 2006) and no studies have evaluated the genetic diversity of its wild populations in Indonesia. Therefore, this research was conducted to analyze the level of genetic variation of teak mistletoe *D. pentandra* using the Random Amplified Polymorphic DNA (RAPD) marker.

MATERIALS AND METHODS

Sampling Sites

The field data gathering and genetic material sampling were conducted at the Padangan teak CSO compartments or blocks, located at 111°34'57.3" E and 07°12'56.1" S and also at Bancer and Payaman Villages, Ngeraho District, Bojonegoro Regency, East Java Province, Indonesia (Fig. 1). The teak CSO area was divided into eight compartments that are further divided into blocks. A total of 132 blocks, at ± 5 ha each totalling to ± 660 ha, were established. Some 144 clones were planted repeatedly in the blocks from 1983 until 1996 (Corryanti 2015). The molecular analysis was carried out at the Genetics and Forestry Molecular Laboratory, Silviculture Department, Faculty of Forestry, IPB University, Indonesia.

Collection of Samples

Leaf samples of *D. pentandra* were randomly collected from five host teaks randomly selected from the observation measure plots (OMP subplots). Four OMPs were established at the observation sample plots (OSP units) (n = 3, 50 x 50 m in size) depending on the level of infestation (light, moderate and heavy) (Muttaqin 2016), but one control OSP plot was not included because there was no infestation or no leaf samples of *D. pentandra* were found, referring to modified EFF or TS/CRC990 (Drescher *et al.* 2016) (Figs. 1, 2a, 2b). The number and distribution of mistletoe leaf samples were collected from the crown layers of each host teak. The crown layers consisted of UU (upper and sub-section upper), UM (upper and sub-section middle), UB (upper and sub-section under), M (middle), and B layer (below). The number of collected samples (n) for each layer were UU (n = 45), UM (n = 57), UB (n = 31), M (n = 56), and B (n = 15) totalling to 2014 leaf samples of *D. pentandra*. That procedure included the use of binoculars, digital cameras, GPS of Garmin Oregon 550 and a map of mistletoe infestation at Padangan teak CSO with scale 1 : 18,000 covering the infestation from years 2010-2014.

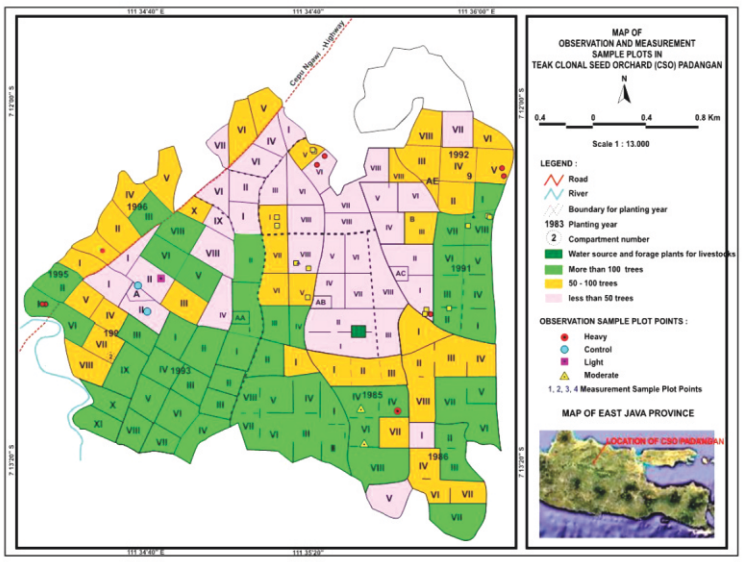

Figure 1 Location map of OSPs at the OMP in Padangan teak CSO (Muttaqin 2016)

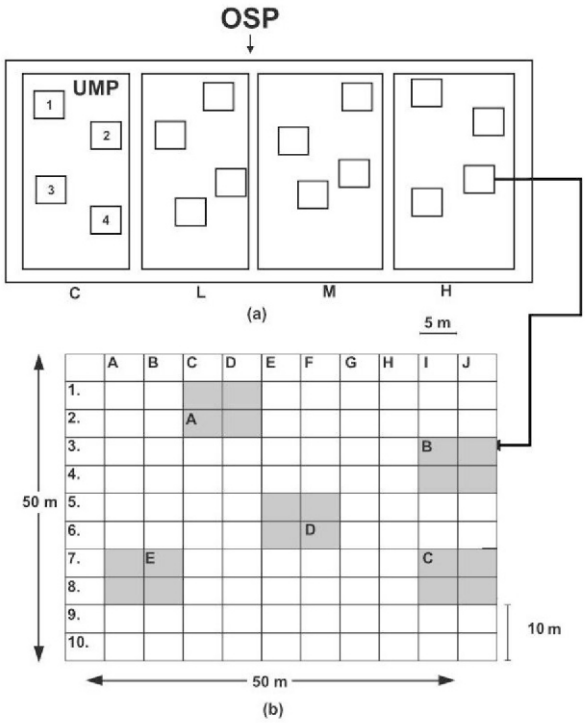

Figure 2 Field layout of the sampling units: (a) Position of the OMPs (n = 4) in the OSP units; (b) Position of the sub-OMPs (n = 5) in an OMP unit (source: EFForTS/CRC990 2012, modified for this research)
Notes: C = control; L = light; M = medium; H = heavy.

Laboratory Procedures

DNA was extracted from leaf samples and isolated using the modified CTAB (Cetyltrimetyl ammonium bromide) method (Doyle 1991; Aritonang *et al.* 2007). DNA quality was carried by PDA (Potato Dextrose Agar) electrophoresis of 1% agarose gel at 100 volts using the buffer TE 50 µL, 3 µL DNA and 2 µL BJ (Blue Juice). The product of electroforesis was given the solution Gelred Tm nucleid acid and photographed on UV transiluminator model TFX-20.LM following Aritonang *et al.* (2007). Five random universal primers, namely; OPP-9, OPP-15, OPP-19, OPBH-20, OPBH-16, and 10 primers used by Amico *et al.* (2014) for *Tristeric corymbosus* (Loranthacea) of same family as *D. pentandra* were used in this research (Table 1).

Table 1 Tested primers and base sequences for the PCR-RAPD analysis of *D. pentandra*

No.	Primers	Base sequences (5' – 3')
1	OPP-9*	GTGGTCCGCA
2	OPP-15*	GGAAGCCAAC
3	OPP-19*	GGGAAGGACA
4	OPBH-20*	CACCGACATC
5	OPBH-16*	CTGCGGGTTC
6	OPA-17	GACCGCTTGT
7	OPB-04	GGACTGGAGT
8	OPB-07	GGTGACGCAG
9	OPB-12	CCTTGACGCA
10	OPB-15	GGAGGGTGTT
11	OPA-04	AATCGGGCTG
12	OPB-18	CCACAGCAGT
13	OPB-20	GGACCCTTAC
14	OPF-11	TTGGTACCCC
15	OPL-12	GGGCGGTACT

Note: * = primer selected for this study (Kissinger 2013; Amico *et al.* 2014).

Table 2 Amplification steps of the PCR-RAPD marker

Stages	Temperature (°C)	Time (minutes)	Cycle
Pre-denaturation	92	5	1
Denaturation	92	1	
Annealing	32; 35	1	35[a]; 45[b]
Extension	73	1	
Final Extension	73	10	1

Notes: [a] PCR machine at MJ Research PTC-100; [b] PCR machine at AB Applied Biosystem VeritiTM Thermal Cycler.

The primers were selected by temperature optimization (annealing) with the PCR process ranging from 32 °C, 33 °C, 34 °C, 35 °C, 36 °C, to 37 °C. Through electrophoresis, the five primers, i.e., **OPP-9, OPP-15, OPP-19, OPBH-20, OPBH-16** showed clear DNA fragment bands at optimization temperature of 32 °C and 35 °C with 35 and 45 cycles (Tables 1 and 2). However, the other primers obtained unclear DNA fragment bands. The primers used were then diluted (5x, 10x) or as ratios 1:50, 1:100 in ddH_2O to elucidate the crystals of DNA bands by electrophoresis as a mixture of materials in a microtube for the PCR-RAPD process. Ingredients in the PCR-RAPD process of 1x reaction comprised of Psd H_2O (2.0 µL), Go Taq® Green Master Mix (6.0 µL), Primer oligonucleic (2.0 µL), DNA template (2.0 µL).

The amplified PCR-RAPD marker was identified by electrophoresis using 2% agarose gel in 50 µL TE buffer, 3 µL DNA, and 2 µL BJ (Blue Juice) for 45 min at 100 volts. The yields of the electrophoresis were given a Gelred Tm nucleic acid solution and were photographed on the UV transilluminator TFX-20. LM model (Nybom *et al.* 2014; Aritonang *et al.* 2007) to identify the clear bands.

The scoring used for locus marker was 1 if having a band and 0 if having no band. Further interpretation of each primer was carried out using the software POPGENE version 1.31 and NTSYSpc version 2.02. POPGENE was also used to compute for other statistics (e.g. allele frequency, gene diversity, genetic distance, F-statistics, multilocus structure. Meanwhile, NTSYS NTSYSpc was used in the cluster analysis of qualitative molecular genetic data (Rohlf 1998; Yeh *et al.* 1999; Aritonang *et al.* 2007).

RESULTS AND DISCUSSION

Genetic Variation

The 204 PCR amplified samples, using the five RAPD markers (OPBH 16, OPBH 20, OPP 9, OPP 15, and OPP 19), produced 46 clear polymorphic DNA bands (loci) with the base length ranging from 50 to 1500 bp in size (Fig. 3). Each primer produced a range of 8 to 11 loci (mean of 9.2 loci), and the polymorphic loci percentage (PLP) ranged from 32.61 to 86.96% (Table 4). These results confirmed those in the study conducted by Poerba and Sunaryo (2006) on the same *D. pentandra* on 22 hosts, excluding teak, at Eka Karya Botanical Garden, Bali. Different from those used in this study, their study used two primers consisting of OPA-11 which produced 12 polymorphic bands (PLP 100%) and OPC-12 which produced 9 bands with 8 polymorphic bands (PLP 88.89%) and 1 monomorphic band. Both primers have bands ranging from 150 to 1700 bp in size. The same primer for predicting the genetic variation of *Nepenthes gracilis* Korth. in Kerangas Forest, Indonesia, produced a band ranging from 150 to 1400 bp in size and PLP of ≥ 82.75% (Kissinger 2013).

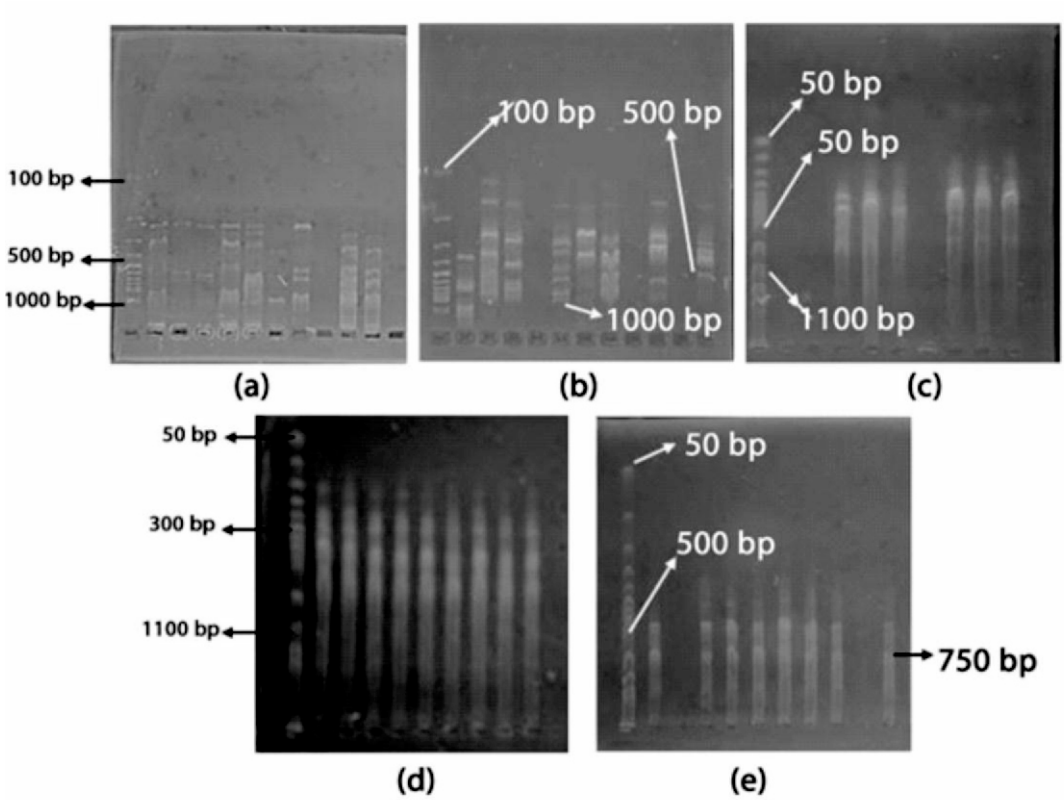

Figure 3 PCR amplification pattern with five markers: (a) OPBH 16, (b) OPBH 20, (c) OPP 9, (d) OPP 15, and (e) OPP 19

Table 3 The primer sequence and number of polymorphic bands

No	Primer	Sequence	Number of polymorphic bands	Base length (bp)
1	OPBH 16	5'CTGCGGGTTC '3	8	50–1500
2	OPBH 20	5' CACCGACATC '3	10	100–1500
3	OPP 9	5' GTGGTCCGCA '3	11	50–1500
4	OPP 15	5' GGAAGCCAAC '3	8	100–1100
5	OPP 19	5' GGGAAGGACA '3	9	50–1100
	Total		46	

Based on the five parameters of genetic diversity (H_e) within population, the highest value (0.255) was obtained by those mistletoes in the UM crown layer and the lowest (0.114) was obtained by those in the B crown layer (Table 4). The higher the H_e, the higher is the Shannon index (I) and the N_e (the sum of effective alleles). In addition, genetic variation of those in the UU, UM, and UB crown layers which have the upper crown sections (H_e = 0.181 – 0.255) are greater than those in the M layer (middle crown) (H_e = 0.227) and B layer (below crown) (H_e = 0.114). This indicated that the dispersal agent of mistletoe seeds, the birds cabai jawa (*Dicaeum trochilium*) and other specialist frugivores, preferred the upper and middle crown sections than the layer below (Muttaqin *et al.* 2016). The birds attached the seeds on branches and twigs through their bird droppings.

The H_e value of *D. pentandra* using the RAPD marker was classified as rather low (0.194). This value was lower than *Tristeric corymbosus* using RAPD marker. Comparison with the different marker, It was lower than *Arcethobium* spp. and *Viscum album* using AFLP marker, also it lower than *Ficus deltoidea* using ISSR marker. However, It be within the range of *V. coloratum* (H_e = 0.032–0.672) using Microsatellite marker (Table 5).

Table 4 Genetic variation using RAPD analysis of mistletoe *Dendrophthoe pentandra* (L.) Miq. at the different crown layers of infected host teak trees at Padangan teak CSO, Indonesia

No	Layer[a] (crown part)[b]	N	PLP (%)	Na	Ne	H_e	I
1	UU	45	60.870	1.609	1.306	0.181	0.273
2	UM	57	86.960	1.870	1.429	0.255	0.391
3	UB	31	65.220	1.652	1.323	0.192	0.295
4	M	56	76.090	1.761	1.377	0.227	0.346
5	B	15	32.610	1.326	1.186	0.114	0.171
Sum		204	321.750	8.218	6.622	0.969	1.476
Average on type level		41	64.350	1.644	1.324	0.194	0.295
Standard deviation (SD)		18	20.430	0.204	0.091	0.054	0.083

Notes: [a] Irawan (2004), modified for this study: N = sum of samples; PLP = Polymorphic Locus Percentage; Na = sum of observed samples alleles; Ne = sum of effective alleles; He = Heterozygosity expectation; I = Shannon Index;
[b] UU = upper crown sub-section upper, UM = upper crown sub-section middle, UB = upper crown sub-section under; M = middle crown, B = below crown.

Table 5 Comparison of genetic variation of mistletoes in this study and other studies using RAPD and other markers

Species	He	Marker	Origin	Source
Dendrophthoe pentandra	0.194	RAPD	Indonesia, Padangan teak CSO	This study
Tristerix corymbosus	0.365	RAPD	Chile	Amico *et al.* 2014
Malaysian mistletoe Fig (*Ficus deltoidea* Jack)	0.500 - 0.750	ISSR	Malaysia	Zimisuhara *et al.* 2015
Viscum album	0.820	AFLP	Korea	Yi *et al.* 2013
Arcethobium spp. Viscaceae	0.238	AFLP	Western North America, Oregon, California	Reif *et al.* 2015
Melampyrum sylvaticum	0.330 - 0.750	Microsatellite	The United Kingdom	Crichton *et al.* 2012
Phoradendron californicum	0.364 - 0.924	Microsatellite	Mexico	Arroyo *et al.* 2013
Viscum coloratum	0.032 - 0.672	Microsatellite	Korea, Japan, China	Kim *et al.* 2017

The 19 novel polymorphic microsatellite markers for mistletoe *Viscum coloratum* have been developed for its use as anti-cancer medicinal plants and have become a novelty in the molecular analysis of mistletoes (Kim *et al.* 2017). Fortunately, cross-species amplification indicated that those markers can also be used for molecular analysis associated with other species coming from the same family (Santalaceae). That result was also tested for cross-species amplification in *V. articulatum* as a potential medicinal plant.

The level of genetic variation of mistletoes play an important role in their capability to adapt to changes in environmental condition, such as lack of water, nutrient, and their regeneration. Low genetic variation implies a high sensitivity to heterogeneous environment that is detrimental to the growth and development of mistletoes. *D. pentandra* has a rather low genetic diversity (Table 4), therefore, it is important to maintain the genetic variation of *D. pentandra* in order to protect its existence and prevent it from extinction, and this requires effective conservation strategies. The massive utilization of *D. pentandra* as a medicinal plant is a cause of concern even as they are hanging on host tree and crops.

Genetic diversity estimates among and within populations of mistletoes species, will further help in the development of effective strategies for their conservation (Kim *et al.* 2017). The knowledge gained from research using Microsatellite markers was needed in designing conservation management programs for *M. sylvaticum* in the United Kingdom (Crichton *et al.* 2012). Such information will also be useful for other related species of endangered non-weedy hemiparasites, according to Mellado & Zamora (2019), with similar traits and life history.

Genetic Distance among Mistletoes on Different Canopy Layers

The largest genetic distance (0.131) was obtained significantly between mistletoes hanging on the UB and B layers which have different crown sections while the shortest genetic distance (0.038) was significantly between those at the UU and UM layers which were at the same upper crown sections (Table 6).

Table 6 Genetic distances between mistletoes hanging on different crown layers of host teaks

Crown layer	UM	M	B	UB	UU
UM	****				
M	0.041	****			
B	0.119	0.100	****		
UB	0.092	0.065	0.131	****	
UU	0.038	0.083	0.124	0.101	****

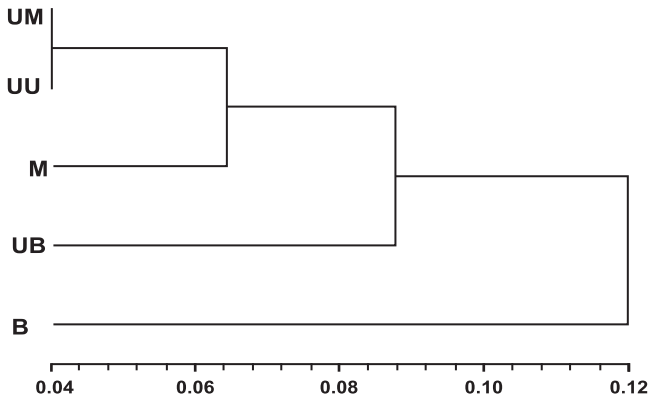

Figure 4 UPGMA dendrogram of the genetic distances, based on RAPD marker, of *D. pentandra* mistletoes growing on the different crown layers of teak
Notes: Crown layers: UU = upper crown sub-section upper, UM = upper crown sub-section middle, UB = upper crown sub-section under; M = middle crown, B = below crown.

The genetic distance or relatedness among mistletoes growing on different crown layers (described by a cluster or a dendrogram) were spread over a Eucledian distance consisting of eight units (0.04 - 0.13) (Fig. 4). Two different clades were identified in which the first clade consisted of mistletoes from the UM, UU, M, UB crown layers, while the second clade only contained those mistletoes in the B crown layer which was considered an outgroup. In this case, the mistletoes in the B layer were more genetically distant from other mistletoes in the UU (0.124), UM (0.119), UB (0.131), M (0.100) (Table 6). Hence, as predicted, the two clusters were of different genetic structure.

Another cluster analysis of 22 *D. pentandra* collected from several different locations had separated the samples into two clusters; one cluster consisted of 3 groups, namely; group 1 (6 collections), group 2 (13 collections), and group 3 (2 collections), whereas the other cluster, the outgroup, consisted of only 1 collection (Poerba & Sunaryo 2006). The Euclidean distance was 43 units (0.490 - 0.920) longer than this study result (0.038 - 0.131). The different clustering is due to the different genetic distant that *D. pentandra* grow on teak and others as host tree on different locations. While in this study, *D. pentandra* grow only on teak of crown layers differently.

CONCLUSION

Based on the RAPD marker, the genetic variation, H_e, of *D. pentandra* mistletoe was rather low (H_e = 0.114 - 0.255). The highest H_e (0.255) was found among the mistletoes of the upper middle layer of the canopy, UM layer, while the lowest H_e (0.114) was among the mistletoes in the layer below the crown (B). The genetic distance in the five layers or sections of teak crown inhabited by *D. pentandra* ranged from 0.038 to 0.131, and was clustered into two clades. The first clade consisted of mistletoes in the UM, UU, M, and UB layers, while the second clade consisted of those mistletoes in the B layer.

For Perhutani, the state-owned forestry enterprise, this study results practically implied the thorough removal of mistletoes by pruning them from the entire sections of the crown, as was done successfully on the dwarf mistletoes of Douglas fir.

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