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Genetic profile, diversity, and relationship within and among families in two generations of *Eucalyptus deglupta x pellita* hybrids in northern Mindanao, Philippines

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ABSTRACT. *Eucalyptus deglupta x pellita* is among the best-performing *Eucalyptus* in many field trials in the country. Aside from growth performance evaluation, assessing the generational genetic potentials through DNA analysis is crucial as inbreeding is known to occur among *Eucalyptus* and to ensure that high genetic diversity is maintained for the next selection cycle. This study assessed the genetic diversity and relationships within and among families in two generations of *Eucalyptus deglupta x pellita* using the Random Amplified Polymorphic DNA (RAPD) analysis. Three independent screening using 15 primers in three parents (F1) and 74 progenies (F2) yielded 490 reproducible bands with maximum value detected in the F2. Banding patterns revealed a monomorphic genetic structure in F1 while polymorphic in F2. Polymorphism displayed high genetic variation in the F2 while $EDP2_{F2}$ demonstrated superior genetic diversity within the family. The dendrograms showed high degree of relatedness in the F2 as depicted by the shrinking genetic distances from 0–15 (F1) to 0–1 (F2). The dendrogram also indicated that $EDP1_{F1}$ may have pollinated the F1 as it clustered together with the F2. Genetic variants and constructed dendrograms suggested that the F1 were relatives while narrowing genetic distance was detected in the F2. This RAPD study concludes the genetic relatedness of the F2 to $EDP1_{F1}$ and highest genetic potential of $EDP2_{F2}$; hence, future selection could be done within this family.

Keywords: dendrogram, half-sib, inbreeding, interspecific hybrid, Random Amplified Polymorphic DNA

INTRODUCTION

Industrial plantation forestry is shifting its global production from the west (*i.e.* North America, Western Europe, Japan) to the east (*i.e.* China, India, Brazil, Indonesia) to meet increasing consumer demand (McEwan *et al.* 2019). This implies the expansion of plantation in Asian countries, including the Philippines. In 2015, the country has about 1.24 M ha of planted forest (FAO 2015). This figure is expected to increase with the ongoing greening effort of the government (FMB 2018) and improving the confidence of investors in forest plantation development (FMB 2009). For instance, some 138,020,616 seedlings were established during the implementation of the National Greening Program (NGP) since 2011 (FMB 2018). Based on Balangue's (2016) field assessment, the 85% survival rate would result to around 117,317,524 trees added to the growing planted forest of the country.

Several publications stated that *Eucalyptus*, together with other exotic trees species, will continue to dominate the global tree plantations in the future (*i.e.* FAO 1979; Cossalter & Pye-Smith 2003; McEwan *et al.* 2019). Among the many advantages of this species are high wood quality, fast-growing ability, the abundance of and access to quality germplasms, and adaptability to a much broader environmental condition (FAO 1979; Ball 1995; Malab 1996; Tolentino 2008). As such, large-scale plantations of the species have been established worldwide such as in China (Turnball 1981) and Brazil (IBA 2015). However, the same effort that begun as trial planting or rehabilitation species, including the subject hybrid in the Philippines during the 1970s remained small-scale, fragmented, and seemingly fixed on field trials at the research level to date (Lizardo 1960; Tagudar & Gianan 1970; Maun 1978; Malab 1996; Razal *et al.* 2004; Pulhin *et al.* 2006; Piñon *et al.* 2013; ERDB 2012, 2017).

Eucalyptus deglupta x pellita is one of the hybrids that have regional economic importance in the Asia Pacific (Davidson 1995). Although some literature discussed the reproductive biology, germplasm characteristics, seed production, and dispersal on either of the pure line species (*i.e.* FAO 1979; Griffin 1989), knowledge about this hybrid's reproductive biology, ecological distribution, phenology, and genetic characteristics are limited. While most *Eucalyptus* exhibit a mixed mating system with outcrossing rates in the wilds averaging 74% across 23 species (Byrne 2008), there is an increasing evidence that self-pollination is common among this species (*i.e.* Griffin 1990; Lloyd & Schoen 1992). Extensive works on the uses, ecological distribution, phenology, including molecular studies on each of the pure lines (*Eucalyptus deglupta* Blume and *Eucalyptus pellita* F. Muell.) have also been reported (*i.e.* PROSEA 1997; Harwood 1998; Widyana *et al.* 2001; Byrne 2008; Orwa *et al.* 2009; Dombro 2010; Brink 2011; Grattapaglia *et al*. 2012).

Morphological appearance does not necessarily reflect the genetic status of tree species (Sale 2011; Grattapaglia 2000). Hence, despite numerous successful performance of this hybrid in many field trials as a plantation species, particularly in Mindanao (Lizardo 1960; Maun 1978; Le 2009), it is important to assess its genetic potential to claim its superiority. Since gene segregation and recombination normally occur in many *Eucalyptus* hybrids (*i.e.* Jadon *et al*. 2014; Chan *et al*. 2019), generational genetic assessment using molecular analysis would help determine the genetic status within and between generations. This knowledge is important to a government's genetic improvement program that intends to produce the required quality planting materials for industrial tree plantations (ERDB 2012, 2013, 2017).

The discovery of Polymerase Chain Reaction (PCR) in 1993 by Kary Mullis (Reamillo *et al.* 2009) resulted in the use of RAPD or Random Amplified Polymorphic DNA analysis. RAPD is a dominant marker that uses short primers (usually 10 base pairs) of arbitrary sequence, which directs the PCR without needing a prior sequence. This marker can detect both the homozygous and heterozygous dominant genotypes, but not the homozygous recessive (Grattapaglia 1994). RAPD is laborious and displays low genetic data per locus. As such, it is prone to difficulty in-band interpretation and hard to obtain amplification using the same reactions (Karp *et al*. 1997; Sudarmonowati *et al.* 2001; Kindt *et al*. 2009). Nevertheless, this marker is simple, requires less amount of reagents, and does not need skilled personnel, high-tech facilities, and equipment (Grattapaglia 1994; Muchugi *et al.* 2008). It is also found transferrable among related species of *Eucalyptus* (Li 2000) while the RAPD profiles that are unique to a genotype can be generated simply and reliably even among clones (Keil & Griffin 1994). As such, this marker has been widely used in various molecular studies of *Eucalyptus*, such as on genetic linkage map construction, clone and DNA fingerprinting, pedigree analysis, and determination of genetic variation (Grattapaglia 1994 and 2000; Keil and Griffin 1994; Nesbitt *et al.* 1995 and 1997; Li 2000). Therefore, this study aims to determine the genetic profile, diversity, and relationship of three half-sib families of *Eucalyptus deglupta x pellita* using the F1 and F2 generations.

METHODOLOGY

Plant material

The three F1 half-sib mother trees of the *Eucalyptus deglupta x pellita* hybrids used in the study were collected from Kitcharao, Agusan Del Norte in Eastern Mindanao. The hybrids were products of natural hybridization between *E. deglupta* (Surigao, Mindanao) and *E. pellita* (Australia) (Dr Cesar Nuevo, personal communication, 15 June 2012). It was originally acquired by the defunct Provident Tree Farms, Incorporated established in 1998 under the Usufruct Program of the Department of Environment and Natural Resources (DENR) Region XIII. The F2 half-sib progenies produced from these three F1 hybrids were part of the 1.2 ha open-pollinated half-sib progeny test, which was established in August 2008 by the Claveria Tree Nursery Inc. (CTNI), in Barangay Gumaod, Claveria, Misamis Oriental (lat. 8.66°N, long. 124.8°E) (**Figure 1**).

It is composed of four different families: one *E. deglupta* (9000 m^2) and three *Eucalyptus deglupta x pellita* hybrids (3000 m^2). The three hybrids were classified as EDP1$_{F1}$, EDP2$_{F1,}$ and EDP3$_{F1}$ (**Table 1**). The area is rolling with the north-east aspect and full light exposure. It has a mean annual rainfall (2170.6 m), mean annual temperature (28.1°C), and mean annual relative humidity (84%) with surrounding trees such as *E. deglupta*, *Falcataria moluccana* [(Miq.) Barneby & J.W. Grimes)], and *Artocarpus blancoi* (Elmer) Merr. Seeds from each parent tree were collected, germinated, and raised to produce the seedlings at CTNI (Piñon *et al*. 2013). From 172 planted seedlings with 51 (EDP1$_{F2}$), 70 (EDP2$_{F2}$), and 51 (EDP3$_{F2}$), 74 surviving half-sib progenies with 25 (EDP1$_{F2}$), 31 (EDP2$_{F2}$), and 18 (EDP3$_{F2}$) were used in the RAPD analysis (**Figure 2**).

Leaf sampling

Leaf sampling for DNA analysis of tropical trees by Muchugi *et al.* (2008) was used during field collection both for the F1 (half-

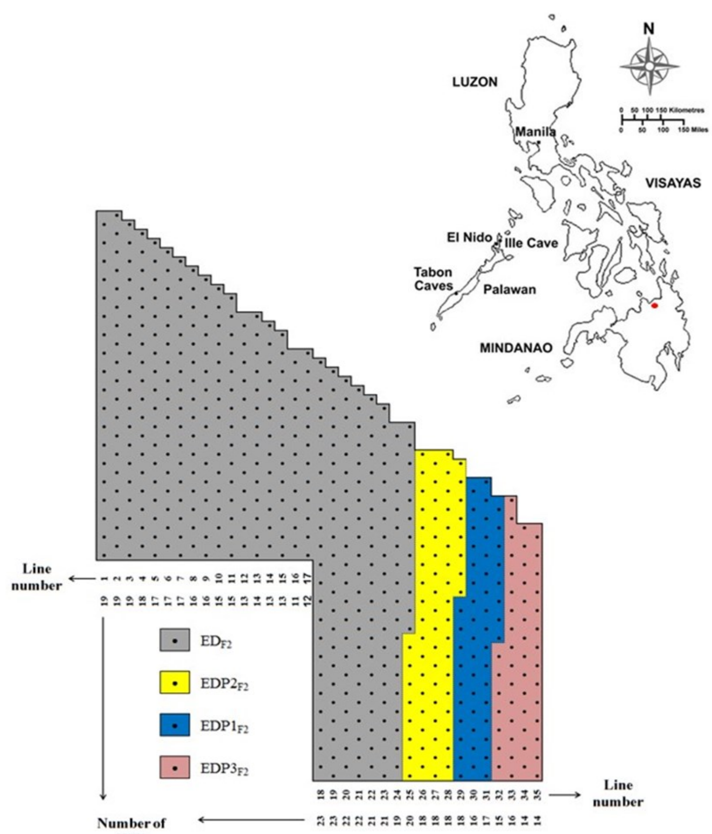

Figure 1. Site map showing the second generation of eucalyptus half-sib progeny test at Claveria Tree Nursery Inc. (CTNI), Claveria, Misamis Oriental (red dot in the Philippine map). It is composed of one family of *Eucalyptus deglupta* (ED$_{F2}$) and three families of *Eucalyptus deglupta x pellita* (EDP1$_{F2}$, EDP2$_{F2,}$ and EDP3F$_2$).

Table 1. Description of the three F1 half-sib mother trees of *Eucalyptus deglupta x pellita*.

Description	$EDP1_{F1}$	$EDP2_{F1}$	$EDP3_{F1}$
from EDP1F1	-	23.9	28.2
from EDP2F1	-	-	5.9
Slope Position	High	Low	Low
Stump Diameter (cm)	50[a]	55[b]	55.7[c]
Total Height (m)	3	11.7	26.9
Merchantable Height (m)	-	-	15.2
Crown Area (m)			
EW	-	-	18.2
NS	-	-	13.8

[a, b]cut in 2010; [c]uncut

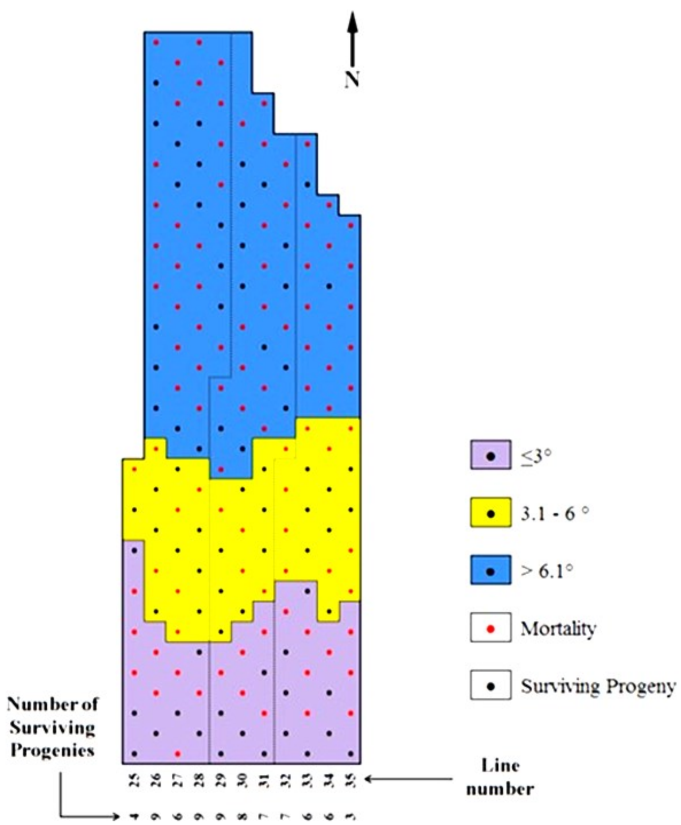

Figure 2. Surviving F2 progenies in various slopes of half-sib progeny test of *Eucalyptus deglupta* x *pellita* hybrid at Claveria Tree Nursery Inc. (CTNI), Claveria, Misamis Oriental.

sib parents) and F2 (half-sib progenies). Leaf samples for each F1 were collected from Kitcharao, Agusan del Norte. While samples of F2 progenies were gathered from half-sib progeny test at the CTNI. Each sample was placed inside a transparent resealable plastic sachet with respective labels. The leaves were packed in a styrofoam container with ice and transported from CTNI to Los Baños, Laguna. Samples were kept in the freezer (-80°C) of the Ecosystems Research and Development Bureau (ERDB) Molecular Laboratory at the University of the Philippines Los Baños (UPLB) until use.

DNA extraction and quantification

Grinding of leaf samples with liquid nitrogen was done at the ERDB Molecular Laboratory, while the rest of RAPD analyses were completed in the Genotyping Services Laboratory (GSL), formerly Molecular Markers Application Laboratory (MMAL) of the International Rice Research Institute (IRRI). This study used a combination of CTAB buffer (cetyl trimethyl ammonium bromide) and SDS (sodium dodecyl sulfate) DNA extraction method used by Keil & Griffin (1994) in the RAPD study for *Eucalyptus* hybrid clone. However, the non-amplifications of reproducible bands after several attempts resulted in some modifications. These changes included the non-use of Ribonuclease A and instead of 2 x CTAB plus 1% SDS, the study applied 2 x CTAB plus 20% SDS based on the DNA extraction method applied by the ERDB Molecular Laboratory on forest tree species (Delos Reyes, personal communication, 20 October 2011). Reagents used were CTAB (pre-heated at 65°C), 20% SDS extraction buffer Chloroform-isoamyl alcohol (24:1), Isopropanol (kept in the freezer), 70 and 95% ethanol, and 1 x TE buffer (1-liter solution is composed of 12.1g Tris, 6.18 g Boric acid, and 0.93 g EDTA). DNA was quantified in 1% agarose gel through electrophoresis while bands were viewed under UV imager (Alpha Imager HP, Cell Biosciences).

RAPD generation

RAPD generation was done for all the primers using the modified protocols from Li (2000) and Nesbitt *et al.* (1995). Modifications included the use of 100 µM instead of 200 µM of each dNTP, 50 µM rather than 200 µM of each primer, Taq DNA Polymerase produced by GSL instead of 1 unit of DyNazyme^{TM} II DNA Polymerase, $MgCl_2$, 10 x PCR reaction buffer with 1.5 $MgCl_2$ rather than 1 x DyNazyme^{TM} II DNA Polymerase buffer, and the use of sterilized nano pure water. In detail, a PCR reaction mixture of 20 µl final volume was used [3µl (20 ng *Eucalyptus* DNA), 2 µl (100 µM of each dNTP), 2µl (50 µM primer), 2 µl (1 unit of Taq DNA Polymerase produced by GSL), 0.8µl (1 µM $MgCl_2$), 2 µl (10 x PCR reaction buffer with 1.5 $MgCl_2$), and 8.2 µl sterilized nano pure water. Fifteen primers, synthesized by Invitrogen, where six (RAPD-01 to 6) and nine (RAPD-07 to 15) sequences were used, as adapted from Keil & Griffin (1994) and Li (2000), respectively. Each reaction was overlaid with one drop of mineral oil (Li 2000). Amplifications were performed using the modified protocol from Keil & Griffin (1994) where the initial denaturation of 1 min at 94°C was followed by 45 cycles of 1 min at 94°C denaturation, 1 min at 38°C annealing, and 2 min at 72°C extension, ending with 5 min at 72°C final extension using the G-Storm PCR machine Model GS1. After adding 2 µl loading dye, samples of 10 µl was loaded into 1% agarose gel (1x TBE buffer with 24 µl of SYBERSAFE). Amplified RAPD fragments were then separated for one hour using 150 V before viewing under the Alpha Imager HP, Cell Biosciences.

Data analysis

Three sets of independent screening for the entire samples were conducted. Each band detected was treated as an independent locus with two alleles of either presence (1) or absence (0). This study adapted the identification and scoring of reproducible bands applied by Keil & Griffin (1994) in various species of *Eucalyptus*. Only reproducible bands in at least two sets of screenings were scored manually and presented in a binary matrix. Using all primers over three screenings, individuals with poor, unclear, and undetected reproducible bands were deleted prior to statistical analysis. RAPD profiles were described using the numbers of yielded reproducible bands; monomorphic, polymorphic, and unique bands. RAPD bands of different molecular weight (M) are interpreted as separate loci (Fritsch & Rieseberg 1996). In this study, monomorphic referred to bands present in all individuals within a family of the same M while polymorphic were those present in at least one, but not all individuals within a family of the same M. Unique bands, on the other hand, were a band(s) present in at least one individual within a family but absent in the other two families of the same M.

Percentages of monomorphism, polymorphism, and unique bands were also computed using the formula of Balasaravanan *et al*. (2005). These were determined using the number of bands amplified per family over the total number of detected bands per generation multiplied by 100%. The diploid data analysis for dominant markers of POPGENE version 1.31 (Yeh *et al.* 1999) with the assumption that Hardy-Weinberg is at equilibrium was likewise used to compute the number of alleles, Nei's (1973) gene diversity, Shannon's information index, number of polymorphic loci, and percent polymorphic loci. The same program was used in the construction of the dendrograms based on Nei's (1978) genetic distances using UPGMA.

RESULTS AND DISCUSSION

RAPD profile

Table 2 summarizes the results of three independent RAPD-PCR analysis within and among three half-sib families in two generations of *Eucalyptus deglupta x pellita* hybrids using 15 primers. **Figure 3** presents the examples of the RAPD profiles with undetected bands using 15 primers in three screenings**.** Bands were viewed approximately from 170 to 1500 base pairs (bp), with the most number of occurrences between 290 to 905 bp. From a total of 74 surviving F2, three were removed before statistical analysis due to poor, unclear, and undetected amplifications. One of each came from $EDP1_{F2}$ (30–15), $EDP2_{F2}$ (26–16), and $EDP3_{F2}$ (34–12). This resulted to 74 samples, including the three individuals of the F2 generation. From a total of 2,499 bands amplified, 490 RAPD bands were found reproducible with a mean of 81.67 bands. Among families, a total of 172 and 318 bands were obtained from the F1 and F2, respectively. Within the F1, $EDP3_{F1}$ registered the highest number of reproducible bands (65), while $EDP2_{F2}$ with 113 bands got the maximum number of bands within the F2.

This study yielded relatively more numbers of reproducible bands than previous RAPD studies on *Eucalyptus* that ranged from 77 to 223 bands (Nesbitt *et al.* 1995; Sale 2011; Li 2000; Fernandez *et al*. 2006). In contrast, scorable bands achieved in the study were relatively less than the 1300 bands generated from a single F1 interspecific family of *Eucalyptus urophylla x grandis* using 137 primers (Verhaegen & Plomion 1996). Apart from numerous primers used, these authors did not mention whether these bands were reproducible or just the total number of amplifications. For instance, from a total of 1,050 bands detected in *Camellia sinensis* (L.) Kuntze using 20 RAPD primers, Chen *et al*. (2005) reported

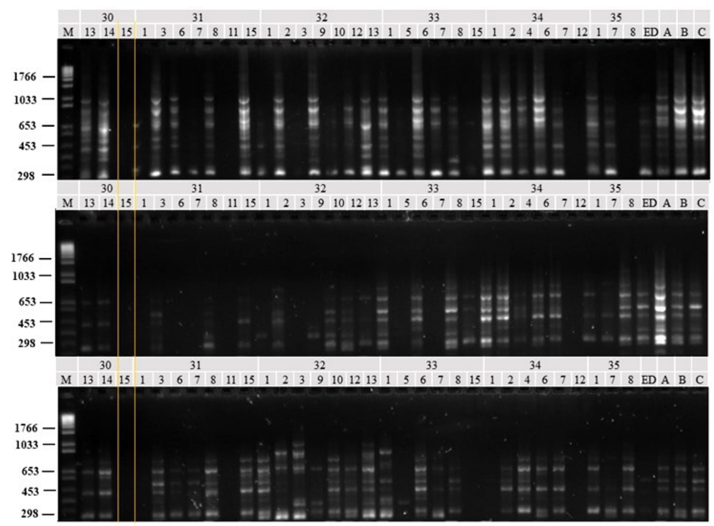

Figure 3. Example of three RAPD profiles amplified using RAPD-04 showing the banding pattern yielded from the F2 (30-13 to 35-8) and the F1 (A, B, and C). M represents the molecular weight in base pair (bp). Note the non-detection of bands in three independent screening in tree number 30-15 (bounded with 2 yellow lines) and 34-12. ED represents the bands amplified from a pure-line eucalyptus known as Bagras (*E. deglupta*), which were included during the RAPD, but not in the analysis for this study.

Table 2. Yielded RAPD fragments in two generations of *Eucalyptus deglupta x pellita*.

Generation	Family	NS	RB	No. of Bands			Percent (%)		
				M	P	U	M	P	U
	$EDP1_{F1}$	1	45	31	10	4	18.02	5.81	2.33
F1	$EDP2_{F1}$	1	62	31	24	7	18.02	13.95	4.07
	$EDP3_{F1}$	1	65	31	27	7	18.02	15.70	4.07
Sub-total		3	172	93	61	18			
Mean[1]		1.5	86	46.5	30.5	9	18.02	11.82	3.49
	$EDP1_{F2}$	23	107	0	105	2	0.00	33.02	0.63
F2	$EDP2_{F2}$	30	113	0	109	4	0.00	34.28	1.26
	$EDP3_{F2}$	18	98	1	96	1	0.31	30.19	0.31
Sub-total		71	318	1	310	7			
Mean[2]		23.67	106.00	0.33	103.33	2.33	0.10	32.49	0.73
Total		74	490	94	371	25	19.18	75.71	5.10
Mean[3]		12.33	81.67	15.67	61.83	4.17	9.06	22.16	2.11
SD[4]		12.99	27.93	16.80	46.01	2.48	9.82	11.88	1.67

that 137 of these bands were reproducible. Although the amount of generated scorable bands varies depending on the number of primers, there is a general trend on the source of DNA materials used for RAPD studies in *Eucalyptus* (*i.e.* wild, natural population, or hybrid). While a lesser number of fragments were detected from clones (Keil & Griffin 1994; Fernandez *et al*. 2006) than those from the wild or natural populations (Nesbitt *et al*. 1995; Li 2000), this was relatively higher when the subject populations were a product of interspecific hybridization (Verhaegen & Plomion 1996).

Gathered scorable bands in the F1 varied from 3 (RAPD–09) to 20 (RAPD–03) per primer and from 13 (RAPD–05) to 28 (RAPD–03) per primer in the F2 (**Table 3 and 4**). The highest number of polymorphic bands (7) within the F1 was amplified using primer RAPD–03 from $EDP3_{F1}$. Together with the RAPD–04, RAPD–03 recorded the highest number of polymorphic bands. $EDP1_{F2}$ got 10 polymorphic bands using these primers among families in the F2. Unlike with the F1, all families in the F2 also registered a similar number of highest polymorphic bands (10) per primer where RAPD –03 (with sequence 5' CAAGCCAGGA 3') was found to have the most number of bands. It can be noted that the same 10-primer sequence was used and found reproducible in other RAPD studies on *Eucalyptus* (*i.e.* Keil & Griffin 1994; Li 2000). This implies that this specific sequence is common to various species of *Eucalyptus*. With the possible inaccessibility to a more powerful tool of assessing the genetic diversity aside from the RAPD, this particular primer

Table 3. RAPD Primers, sequences, and banding patterns obtained from the F1 generation of *Eucalyptus deglupta x pellita.*

Primers	Sequences	$EDP1_{F1}$			$EDP2_{F1}$			$EDP3_{F1}$		
		PB	MB	UB	PB	MB	UB	PB	MB	UB
RAPD-01	5' GGATCTCGAC 3'	-	1	-	1	1	1	1	1	-
RAPD-02	5' GCGTTCCATG 3'	-	4	-	1	4	-	2	4	1
RAPD-03	5' CAAGCCAGGA 3'	4	2	-	-	2	-	7	2	3
RAPD-04	5' GGATCTCGA 3'	-	5	-	1	5	1	1	5	1
RAPD-05	5' CAGTTGCGA 3'	1	1	1	1	1	1	-	1	-
RAPD-06	5' ATCGGAAGG 3'	1	1	1	1	1	-	2	1	1
RAPD-07	5' CCCTACCGAC 3'	-	1	-	5	1	-	5	1	-
RAPD-08	5' CTGATGCGTG 3'	-	2	-	-	2	-	-	2	-
RAPD-09	5' CAGACAAGCC 3'	-	1	-	-	1	-	-	1	-
RAPD-10	5' AGCCGTGGAA 3'	1	4	-	1	4	-	-	4	-
RAPD-11	5' GGATCTCGAC 3'	-	1	-	2	1	-	3	1	1
RAPD-12	5' GGACGGGTGC 3'	-	2	-	1	2	-	1	2	-
RAPD-13	5' GAAACGGGTG 3'	-	2	-	2	2	1	1	2	-
RAPD-14	5' CCACGACGAT 3'	-	2	-	6	2	3	3	2	-
RAPD-15	5' GTGACGTAGG 3'	3	2	2	2	2	-	1	2	-
	TOTAL	10	31	4	24	31	7	27	31	7

PB – Polymorphic bands; MB – Monomorphic bands; UB – Unique bands

Table 4. RAPD Primers, sequences, and banding patterns from the F2 generation of *Eucalyptus deglupta x pellita.*

Primers	Sequences	$EDP1_{F2}$		$EDP2_{F2}$		$EDP3_{F2}$		
		PB	UB	PB	UB	PB	UB	MB
RAPD-01	5' GGATCTCGAC 3'	5	-	7	1	4	-	-
RAPD-02	5' GCGTTCCATG 3'	8	-	9	-	9	-	-
RAPD-03	5' CAAGCCAGGA 3'	10	-	8	-	10	-	-
RAPD-04	5' GGATCTCGA 3'	10	-	10	-	8	-	-
RAPD-05	5' CAGTTGCGA 3'	3	-	5	1	4	-	-
RAPD-06	5' ATCGGAAGG 3'	6	-	8	-	4	-	-
RAPD-07	5' CCCTACCGAC 3'	9	-	8	1	8	-	-
RAPD-08	5' CTGATGCGTG 3'	5	-	4	-	6	1	-
RAPD-09	5' CAGACAAGCC 3'	6	1	5	-	3	-	-
RAPD-10	5' AGCCGTGGAA 3'	7	-	9	-	8	-	-
RAPD-11	5' GGATCTCGAC 3'	7	1	6	-	6	-	-
RAPD-12	5' GGACGGGTGC 3'	6	-	7	1	4	-	-
RAPD-13	5' GAAACGGGTG 3'	8	-	8	-	8	-	-
RAPD-14	5' CCACGACGAT 3'	8	-	8	-	8	-	-
RAPD-15	5' GTGACGTAGG 3'	7	-	7	-	6	-	1
	TOTAL	105	2	109	4	96	1	1

PB – Polymorphic bands; MB – Monomorphic bands; UB – Unique bands with a specified sequence can be used in future studies on *Eucalyptus* to lessen the amount of time and resources used in tedious primer screening. This is useful with the recent trend of conducting a single study that utilizes more than one molecular marker (*i.e.* Carlier *et al*. 2008; Sale 2011).

Polymorphism

The genetic structure of the F1 was found monomorphic with total monomorphic bands (93) higher than polymorphic bands (61) (**Figure 4**). In contrast, the polymorphic genetic structure was observed in the F2 with only one monomorphic band and 310 polymorphic bands. Between generations, these obtained a 94 monomorphic bands with a mean of 15.67 bands and a mean percent monomorphism of 9.06% in two generations. From the total monomorphic bands, 93 were amplified using 15 primers in three families of the F1. Each family showed the same number of monomorphic bands (31) and percent monomorphism (18.02%). On the other hand, only the RAPD–15 primer detected a single monomorphic band in the F2. The band was amplified in the EDP3$_{F2}$ with 0.31% monomorphism. Meanwhile, from a total of 371 detected polymorphic bands with a mean of 61.83 bands, more than five-fold (310 bands) were found among families of the F2 than those of the F1 (61 bands). The maximum number of polymorphic bands in the F1 was obtained from the EDP3$_{F1}$ (27 bands) while it was in the EDP2$_{F2}$ (109 bands) for the F2. Consequently, these families achieved the highest percent polymorphism in two generations with 15.70% in the former and 34.28% in the latter.

The detected polymorphism in two generations of the subject *Eucalyptus* hybrids revealed two important findings. First, the significant increase in the number of polymorphic bands as opposed to almost non-detection of monomorphic bands in the F2 compared to F1 suggests a high degree of genetic diversity in progenies of interspecific hybrid. Such a significant increase in variation occurred because hybridization increases heterozygosity, creates new genetic recombination, and promotes genetic segregation (Jadon *et al*. 2014; Chan *et al*. 2019). Second, the calculated percent polymorphism within and among families in two generations implies that tree-to-tree distances, breeding systems, and the source (wild or plantation) of plant materials where DNA samples for RAPD were collected may have affected the resulting genetic variation.

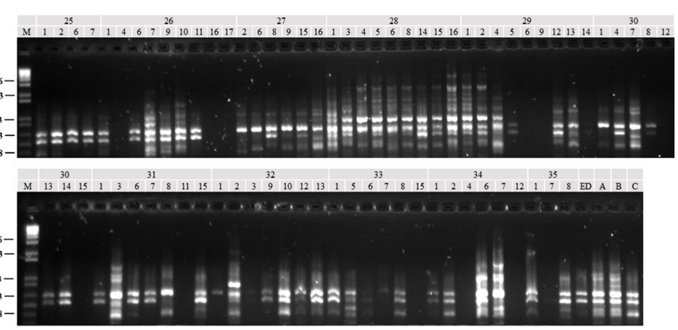

Figure 4. Upper and lower RAPD profiles showing the banding pattern generated using the primer RAPD-15 from the F1 and F2 generations. For the F1, A (EDP3$_{F1}$), B (EDP2$_{F1}$) and C (EDP1$_{F1}$), while for the F2, EDP1$_{F2}$ [(29-1 to 29-9) + (30-1 to 30-15) + (31-1 to 31-15) + (32-9 to 32-13)], EDP2$_{F2}$ [(25-1 to 25-7) + (26-1 to 26-17) + (27-2 to 27-16) + (28-1 to 28-16) + (29-12 to 29-14) and EDP3$_{F2}$ [(32-1 to 32-3) + (33-1 to 33-15) + (34-1 to 34-12) + (35-1 to 35-8)]. Note the monomorphic bands observed in all of the F1 at approximately 453 bp where most of the F2, particularly those trees coded from 27-2 to 28-4, displayed the same banding pattern. M represents the molecular weight in bp. ED represents the bands amplified from a pure-line eucalyptus known as Bagras (*E. deglupta*), which were included during the RAPD, but not in the analysis for this study. Cells on tree numbers 26-16, 30-15, and 34-12 have undetected bands in three screenings. Others were found reproducible in the other two analyses.

In this study, the DNA materials used for the F2 were collected from plantations of three F1 with distances ranging from 5.9 m to 28.2 m apart (**Table 1**). In the pollen dispersal study on *Eucalyptus caesia* Benth. using a paternity assignment conducted by Bezemer *et al.* (2016), they discovered that 74% of the pollen were successfully dispersed and fertilized trees with more than 20 m apart. This signifies that cross-pollination among three F1 families was likely to be high given its relative distances on the ground such that a higher degree of polymorphism was revealed in the F2. Aside from tree-to-tree distances, other factors affect the mode of fertilization and the rate of self or cross-pollination, particularly in *Eucalyptus* (FAO 1979; Griffin 1990; Lloyd & Schoen 1992). There is a need to deepen understanding of these factors and its interactions, particularly on *Eucalyptus deglupta x pellita*.

A large amount of genetic variation was also encountered from outcrossing trees when planted in areas conducive for cross-pollination (Grattapaglia 2000; Grattapaglia & Kirst 2008). Numerous authors claim that *Eucalyptus* are predominantly outcrossing but may also undergo self-pollination (*i.e.* FAO 1979; Griffin 1989; Griffin 1990), especially when contributory factors for outbreeding is limited (Ballesta *et al.* 2015).

It likewise appears that the source (wild, plantation, hybrids) of DNA materials were affecting the percent polymorphism. The mean percent polymorphism of 32.49% in this study was lower than the other RAPD studies with percent polymorphism ranging from 92 to 100% (Nesbitt *et al.* 1995; Sale 2011). However, the referred RAPD studies collected DNA samples from wild populations rather than plantations. In comparison, apart from samples collected from a plantation, DNA materials used for RAPD by Verhagen & Plomion (1996) were a product of reciprocal recurrent selection (RRS). As such, these achieved a relatively lower percent polymorphism (41%) compared to those collected from the wild but relatively higher than this study, particularly in the F1(33.33%). This indicates that higher percent polymorphism could be achieved from *Eucalyptus* hybrids that are products of RRS; although there is a caution as RRS also has negative consequences. For instance, while RRS increases the attainment of desired phenotypic traits with economic importance, it narrows down the genetic diversity (Namkoong *et al.* 1988; Leroy *et al.* 1997; Santos *et al*. 2005; Alves *et al*. 2015). Although slightly higher than this study, Li (2000) obtained low percent polymorphism (46%). In this case, DNA samples were collected from the wild but came from widely separated locations. This suggests that fragmented populations also affect genetic variation. Geographic isolation promotes lesser connectivity, inbreeding depression, and could block the gene flow among populations (Ballesta *et al*. 2015; Schierenbeck 2016) with a more intense effect where tree density is naturally low (Silva *et al*. 2010).

These findings explain why despite a large number of monomorphic bands in F1, polymorphic bands prevailed in F2. Although such variation could be used as an effective tool for conservation (Chan *et al.* 2019), it is not necessarily advantageous for commercial plantation as resultant progenies of interspecific hybrids are prone to inferior quality and produces non-essential genotypes (Subashini *et al.* 2014). Further research is also needed to evaluate the extent of variation since this study only considered the second generation. Although some studies claimed that unchanged and high levels of genetic diversity persist in three generations of *Eucalyptus urophylla* S.T. Blake despite heavy artificial selection (Lu *et al.* 2018a, 2018b).

The significant number of polymorphic bands in the F2 with lower monomorphic and unique bands may suggest the possible occurrence of segregation distortion (SD) or meiotic drive. This phenomenon was found to occur in many F2 progenies of hybrid plants, animals, and trees after the discovery of linkage map construction using various molecular markers, including the RAPD (Kozielska *et al.* 2010). SD, defined as deviation of the observed genotypic frequencies from the Mendelian value (Lu *et al.* 2002), has been previously detected in agricultural crops (Mangelsdorf & Jones 1926; Lu *et al.* 2002; Xian-liang *et al.* 2006) and recently been discovered in hybrid trees, notably in *Eucalyptus* (Verhaegen & Plomion 1996; Bundock *et al.* 2000; Freeman *et al*. 2006; Rosado *et al.* 2011; Bodenes *et al*. 2016). While it can be assumed that SD may exist on the subject hybrid, this remains an assumption unless a similar study is done using the same species.

Unique bands

From the 25 unique bands gathered, 18 and 7 bands were recorded in the F1 and F2, respectively (**Table 2**). The same number of unique bands (7) were recorded both in the EDP2$_{F1}$ and EDP3$_{F1}$ within the F1, while EDP2$_{F2}$ obtained the greatest number of unique bands within the F2. Between generations, both EDP1$_{F1}$ and EDP2$_{F2}$ registered a similar number of unique bands (4). Mean percent unique bands in the F1 (3.49%) were found relatively higher than those computed in the F2 (0.73%). These findings were consistent with other RAPD studies on *Eucalyptus* (de Lange *et al*. 1993; Keil & Griffin 1994; Fernandez *et al*. 2006), which also discovered the importance of unique bands in characterizing, identifying the miss-labeled, and selecting the freezing resistant *Eucalyptus* clones. Unique bands were also found useful not only in trees, like *Eucalyptus*, but also in various plants (*i.e.* Fei *et al.* 2014; Konzen *et al.* 2017; Abdelmigid & Morsi 2018). It is interesting to note that not a single unique band identified in the F2 matched with those bands amplified in the F1. This suggests that such unique bands probably came from another parent *Eucalyptus*, aside from the three half-sib parents considering that natural populations of *E. deglupta* were found in the surroundings of the subject F1.

Genetic diversity

Within a family, EDP2$_{F2}$ showed high genetic potential among the other F2 in all computed genetic parameters, particularly on Nei's (1973) gene diversity (*H'*) (**Table 5**). Calculated H' value within the F2 ranged from 0.20 (EDP1$_{F2}$) to 0.26 (EDP2$_{F2}$) with an average of 0.24. Gene diversity within the population in this study was slightly higher than those obtained from a widely separated population of *Eucalyptus microtheca* F. Muell. (0.17–0.23) using RAPD (Li 2000) and *Eucalyptus cladocalyx* F. Muell. (0.11–0.26) using ISSR (Ballesta 2015). Aside from these species, declining genetic diversity with increasing distance was also observed in *Eucalyptus globulus* Labill. (Jones *et al*. 2013). These indicate that the genetic variation of *Eucalyptus* in geographically isolated areas is affected since pollen dispersal is restricted by distance.

Table 5. Pooled genetic data obtained from the generated RAPD profiles using 15 primers within and among three F2 families of *Eucalyptus deglupta x pellita.*

Parameters	Within			Among (EDP$_{F2}$)
	EDP1 $_{F2}$	EDP2 $_{F2}$	EDP3 $_{F2}$	
Sample size (n)	23	30	18	71
Observed no. of alleles	1.89	1.92	1.81	2
Nei's (1973) gene diversity (mean)	0.2	0.26	0.25	2.47
Shannon's information index (mean)	0.32	0.4	0.39	0.39
Number of polymorphic loci	105	109	96	118
Percent polymorphic loci (%)	88.98	92.37	80.51	100

The high values of genetic parameters for EDP2$_{F2}$, particularly on *H'* value suggests a wider selection option for future genetic improvement from this family. Since trees with high genetic diversity correspond to greater phenotypic variation (Zobel & Talbert 1984; Kitzmiller 1990; Hartl & Clark 2007), it provided more options during plus-tree selection. However, caution must be observed not to rely solely on the result of molecular characterization when establishing a tree improvement program. High values of genetic parameters do not often necessarily mean better phenotypic quality. For example, in the growth performance evaluation on the subject hybrid conducted by Piñon *et al.* (2013), EDP2$_{F2}$ has the lowest mean values obtained on diameter at breast height (DBH), total height and volume compared to the other F2 families, despite showing high genetic diversity. However, since the subject F2 progenies were still at an early stage (33 months), the result may vary when it reaches maturity where desirable phenotypic traits are more fully expressed (Bouvet *et al.* 2002; Wu *et al.* 2012) and when the effect of inbreeding depression (ID) is more significant (Hardner & Potts 1995; Silva *et al*. 2011). For instance, multiple regression analysis revealed significant and positive correlations both between three inter-simple sequence repeats (ISSR) markers and height, and between ISSR marker and DBH in 25-year-old *Copernicia prunifera* (Mill.) H.E. Moore (Fajardo *et al.* 2018), suggesting that as the age progressed, the clearer genetic and phenotypic traits association is observed. The overall mean of Nei's (1973) genetic diversity index (*H'*) value of 2.47 among families compared to an average of 0.24 within the F2 also revealed a ten-fold increase in genetic diversity among families (**Figure 5**). This indicates that greater genetic diversity is feasible for the next cycle of selection when the best individuals across families are selected.

Dendrogram

The first dendrogram (**Figure 6a**) revealed the separation of EDP1$_{F1}$ from the two other half-sib parents, indicating a closer genetic relationship of EDP2$_{F1}$ and EDP3$_{F1}$. The second dendrogram (**Figure 6b**) demonstrates much closer affinities

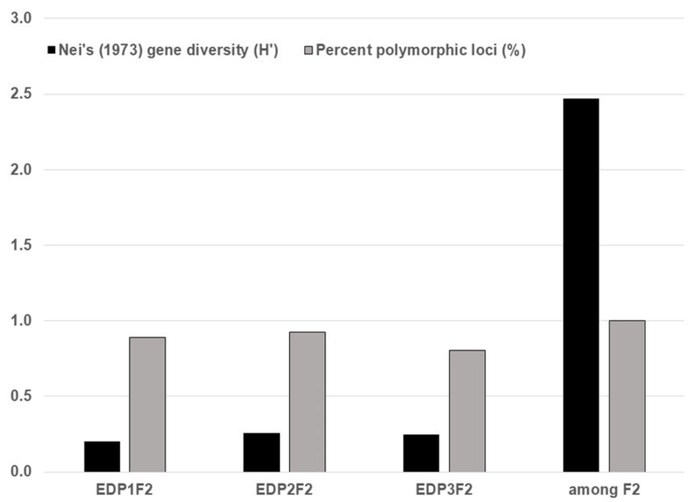

Figure 5. Genetic diversity and percent polymorphic loci within and among F2 half-sib progenies of *Eucalyptus deglupta x pellita* detected using RAPD profiles and analyzed using POPGENE 1.31 version. Values for percent of polymorphic loci were divided by 100. Note the sudden increase in genetic diversity among families, despite having relatively the same genetic diversity within family.

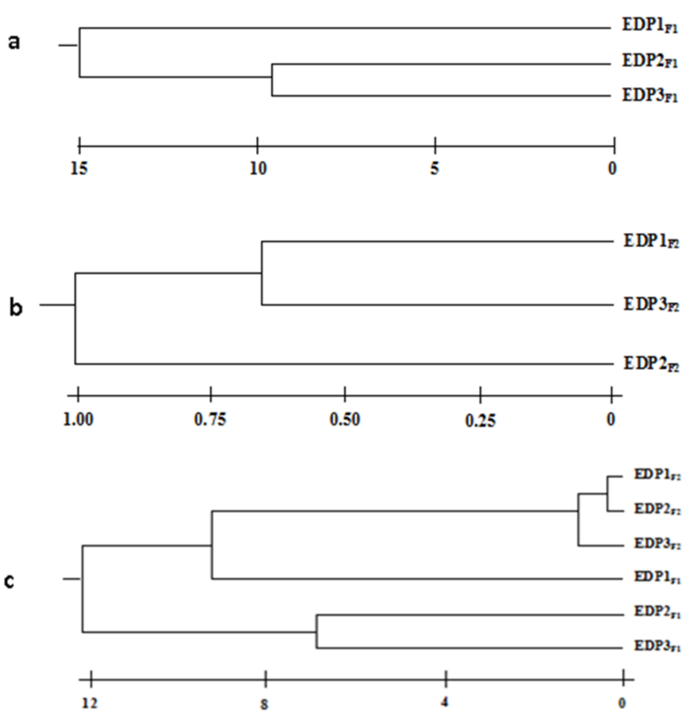

Figure 6. Constructed dendrograms showing the genetic relationships of three F1 (a), three F2 (b), and combined F1 and F2 (c) generations of *Eucalyptus deglupta x pellita* families obtained from detected RAPD fragments. The scale indicates genetic distance.

among F2 compared to F1. The clustering EDP1$_{F2}$ and EDP3$_{F2}$ revealed a closer genetic relationship than the other group. The shrinking of the genetic distance with the scale from 0–15 (F1) to 0–1 (F2) was likely due to a high degree of inbreeding (self-pollination or cross-pollination among relatives) among the F1, which probably resulted to the occurrence of ID in the F2. This suspected ID was found affecting the growth and survival of the subject hybrid based on its phenotypic study (Piñon *et al*. 2013).

The estimated overall mean phenotypic performance in all economically important traits, 33 months after planting indicates the occurrence of ID in three *Eucalyptus deglupta x pellita* under half-sib progeny test. However, although selfing (Griffin 1989) is one possible reason for ID among *Eucalyptus*, it was difficult to specify the main causes or whether ID did occur at least in the subject hybrid. Controlled pollination is needed to discriminate the mode of inbreeding (*i.e.* selfing, kin-mating, outbreeding, etc.), which was not done by this study. Some research proved the negative effect of ID in traits with economic importance (*i.e.* height, dbh, survival) among *Eucalyptus* (*i.e.* Hardner & Potts 1995; Silva *et al.* 2010; Griffin *et al*. 2019) but instead of lowering the genetic diversity, Klapste *et al.* (2017) discovered that it did the opposite in *Eucalyptus nitens* (H. Deane & Maiden) Maiden.

Despite outperforming the other families in various genetic parameters (**Table 5**), EDP2$_{F2}$ obtained the lowest phenotypic performance (Piñon *et al*. 2013). The closest parent tree among the F1 with the F2 progenies was shown on the third dendrogram (**Figure 6c**). Here, all the F2 clustered together with the EDP1$_{F1}$. This suggests that although parent EDP2$_{F1}$ and EDP3$_{F1}$ are physically closer on the ground (**Table 1**) and with higher genetic affinity as shown in the first and last dendrogram (**Figure 6a** and **Figure 7**), it appears that EDP1$_{F1}$ produced and dispersed the most compatible pollen on time that pollinates the receptive flowers of the F1. As explained by Griffin (1989), the time and duration of flowering vary within and between species of *Eucalyptus*, as these are exotics suggesting that appropriate synchrony, timing, and duration of flowering influence the genetic structure of resulting progenies.

Meanwhile, the fourth dendrogram provided more detailed information about the genetic relationships within and between two generations (**Figure 7**). Thirty-seven individuals of the F2 were grouped with F1 and formed cluster 1 while the remaining progenies grouped and formed cluster 2. It appears that some of the pollen that fertilized F1 probably came from the other wild population of *Eucalyptus*, hence, two distinct clusters were constructed. *E. deglupta* was among the trees growing within proximity of F1. This species produce flowers as early as six months (FAO 1979), implying its active reproductive ability that may have pollinated the three F1. Further investigation is required to ascertain this assumption. Apart from confirming the possible occurrence of ID, the last dendrogram demonstrated the genetic affinities among the F2, particularly among the EDP2$_{F2}$ where 57% of its progenies grouped in cluster 1. This suggests that high percent polymorphism and genetic diversity does not necessarily imply greater genetic distance, at least in the subject hybrid. In this study, EDP2 $_{F2}$ achieved the highest value in all genetic parameters among the F2 but displayed the lowest phenotypic performance (Piñon *et al.* 2013) and showed the smallest genetic distance as presented in the cluster 1 of the fourth dendrogram. In comparison, EDP1$_{F2}$ failed to outperform the other F2 in terms of genetic parameters but was found the most widely spread in the last dendrogram. Such that EDP1$_{F2}$ recorded the highest mean values in most of the traits tested (percent survival, DBH, and volume) phenotypically (Piñon *et al.* 2013).

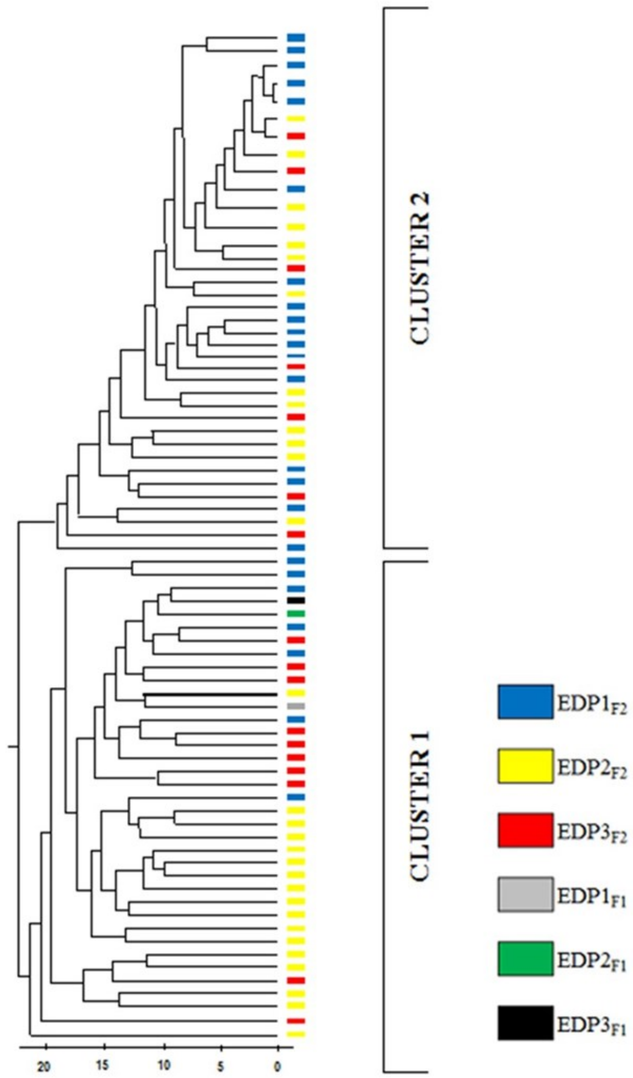

Figure 7. Dendrogram showing the genetic relationship among the three F1 and seventy-one F2 of *Eucalyptus deglupta x pellita*. The scale indicates genetic distance.

CONCLUSIONS AND RECOMMENDATIONS

The RAPD analysis proved useful in assessing the genetic profiles, diversity, and relationship within and among families in two generations of *Eucalyptus deglupta x pellita* hybrid. Using 15 primers, the study amplified greater numbers of reproducible bands than most of the previous RAPD studies on *Eucalyptus*. Comparing the scored bands between F1 and F2 revealed a monomorphic genetic structure of F1 while polymorphic in F2. Calculated polymorphism agreed with other studies that proved the presence of high genetic variation in progenies of interspecific hybrids due to genetic recombination and segregation. Results also showed that computed polymorphism was affected by tree-to-tree distance, breeding system, and source of DNA samples, which paralleled with other genetic *Eucalyptus* studies. Aside from these, numerous factors affect the mode and rate of fertilization. Further study on whether the subject hybrid undergoes self and/or cross-pollination is suggested. Estimated genetic diversity confirmed the relatively monomorphic genetic structures within a family while polymorphic structures among F2. Constructed dendrograms also showed high degree of genetic relatedness among F2 as shown by shrinking genetic distances in F2 compared to F1. The dendrogram further pointed EDP1$_{F1}$ as the possible main source of pollen that fertilized F1 as it established a closer genetic distance and clustered together with all F2.

All genetic variants used, including the constructed dendrogram, indicated that F1 was relative while narrowing genetic distance was detected in F2. Inbreeding depression may also have occurred in F2, which was confirmed in the result of phenotypic performance evaluation. However, this observation may only be considered as an indication since controlled pollination was not done to discriminate the mode of inbreeding and directly confirmed the presence of inbreeding depression. As such, it is recommended for future studies. Finally, RAPD study on *Eucalyptus deglupta x pellita* revealed the high genetic potential of EDP2$_{F2}$ among the second generation, proved the narrowing of genetic distance from first to second generation and shown genetic relatedness of the second generation to EDP1$_{F1}$. These findings are useful for both the government (*i.e.* NGP) and the private sector who are investing in tree plantations. This study argues the importance of diversifying seed sources and avoiding germplasm collection from a limited number of population to prevent related trees with closer genetic distance and ensure the establishment of productive *Eucalyptus* plantations.

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