Malays. Appl. Biol. (2015) 44(4): 73–80

RESEARCH NOTE

GENETIC DIVERSITY OF *Eurycoma longifolia* JACK USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKER IN FOREST RESERVE OF KENEGERIAN RUMBIO, INDONESIA

ROSMAINA, RACHMAT ASHARI and ZULFAHMI*

Department of Agrotechnology, Faculty of Agriculture and Animal Science,
State Islamic University of Sultan Syarif Kasim Riau, Panam,
PO BOX 1004, Pekanbaru 28293, Riau, Indonesia
Tel. +62761-562051, Fax +62761-562052
**Email: fahmiuin@gmail.com*

Eurycoma longifolia Jack is known as pasak bumi in Indonesia, and belongs to the family of Simaroubaceae. *E. longifolia* is only found in Sumatra and Kalimantan forests at altitudes below 700 m above sea level. It is a small tree reaching up to 15 m in height and 15 cm in diameter. This species is grows well in acid, sandy, and low nutrient soils and is often found in beach forests, primary and secondary forests, mixed dipterocarp forests and also in heath forests (Hadiah, 2000). *E. longifolia* is an economically important plant as a source of biologically active compounds for herbal medicine. The ingredients are concentrated in the root, and when extracted can be used as antimalarial compound (Chan *et al.,* 2005), to inhibit growth of human breast cancer cell line (Tee & Azimahtol, 2005), to increase testosterone level (Tambi *et al.,* 2011), to protect bone calcium loss (osteoporosis) (Effendi *et al.,* 2012), and as insecticide (Lina *et al.,* 2009).

Currently, *E. longifolia* has been declared as a protected species in Indonesia based on Regulation of Ministry of Agriculture No. 511/Kpts/PD.310/9/2006, as over collection of this species in the wild has almost caused its extinction from the forest. Therefore, the efforts to conserve the genetic resource of *E. longifolia* have become a priority. Forest reserve of Kenegerian Rumbio in Riau province – Indonesia is one of the target populations for conservation of *E. longifolia*. Information on overall genetic variation in the target population, including the level of variation and genetic relationship of individuals are required for designing an effective conservation strategy. Based on information from local community, there are two types of *E. longifolia* in the forest reserve of Kenegerian Rumbio, reffered to as female and male *E. longifolia*. Local community distinguish both species based on petiole color, e.g. brown reddish and green for female and male *E. longifolia*, respectively. Besides that, if piece of bark or root is licked, female *E. longifolia* will be more bitter than male *E. longifolia*. Although both types can be morphologically distinguished, however, there are no information whether both types are genetically different or not. This information is actually needed for helping forest manager to identify the appropriate species for plantation and conservation purposes. To answer this question, we analyzed genetic variation of two types of *E. longifolia* using random amplified polymorphic DNA (RAPD) markers.

RAPD markers has been known as one of the most commonly used techniques for estimating the genetic diversity in plant populations due to their advantages compared to other DNA molecular techniques such as its simplicity, speed, low cost, small amounts of target DNA required as well as no need for prior sequence information on the target species and radioactivity free procedure (Welsh & McClelland, 1990; Williams *et al.,* 1990; Spooner *et al.,* 2005; Weising *et al.,* 2005; Muchugi *et al.,* 2008). On the other hand, RAPD have some limitations, such as in dominant mode of inheritance with several strategies put forward to increase its informativeness in genetic variation analysis (Lynch & Milligan, 1994). Sometimes, RAPD is considered to have poor reproducibility. However, this weakness can be resolved by optimizing the reaction conditions (Weising *et al.,* 2005; Muchugi *et al.,* 2008). Lynch and Milligan (1994) stated that although this marker has some limitations it can provide valuable genetic variation data within and among populations of plant species.

* To whom correspondence should be addressed.

The objective of this study was to assess the genetic variation and genetic relationship of male and female *E. longifolia* using RAPD markers.

Young leaves of male and female *E. longifolia* were collected from the forest reserve of Kenegerian Rumbio in Riau province-Indonesia. For each type, three individuals sapling were collected so that distance among individuals was more than 50 m. Samples were stored in a freezer at -20ºC until DNA was extracted. Good quality DNA was obtained by Cetyl-Trimethyl Ammonium Bromide (CTAB) method as described by Doyle and Doyle (1990). These DNA samples were further diluted and stored in freezer at -20ºC until RAPD analysis was performed.

Twenty random primers were initially screened to determine the suitability of each primer for this study. Out of twenty primers used, eight primers showed no DNA amplification whereas twelve primers showed DNA amplification. Out of these twelve primers, ten primers were selected as they yielded strong, intense and unambiguous bands. These primers were used for further analysis. DNA amplification was carried out in CFX 96TM Real Time DNA engine Thermal cycler (BioRad). The amplification was programmed as follows: initial denaturation for 5 minutes at 95ºC, followed by 36 cycles of denaturation for 1 minute at 94ºC, annealing for 1 minute at 37ºC, extension for 1 minute at 72ºC, and final extension for 8 minutes at 72ºC. The PCR reaction comprising 15 µl, contained 2.0 µl DNA template (5-10 ng), 1.8 µl primer (5 pmol/µl), 2.2 µl water free RNase, 1.5 µl coral load and 7.5 µl HotStar Taq Master Mix (Qiagen). PCR products were separated in a 1.0% (w/v) agarose gel in 1X TAE buffer at 100 volts for about 30-45 minutes, and then gels were stained in 0.5% (v/v) ethidium bromide solution. The banding patterns of gel were observed under UV light apparatus and photographed using Gel Doc system (BioRad). A 100 bp DNA ladder (Amresco) was included in the gels as a size reference or molecular weight standard. Analysis of banding patterns was performed using an Image Lab version 2.0.1 (BioRad) Software.

Amplification products were scored as '1' for presence and '0' for absence of each band in individual lanes. The scores were entered as a binary matrix for analysis. The genetic diversity was estimated in two ways: (i) percentage of polymorphic loci (PPL), that was calculated by dividing the number of polymorphic bands at the population level by the total number of bands surveyed, and (ii) Shannon's Diversity Index (H'- denotes the diversity of RAPD markers) (Lewontin, 1972: $H' = - \Sigma\, pi \log_2 pi$, where *pi* is the frequency of a given RAPD band). Shannon's diversity index is applicable to analysis of RAPD data because it is relatively insensitive to the bias produced by failures to detect heterozygous individuals (Dawson *et al.,* 1995). Calculation of these two diversity estimates were performed using the software of POPGEN version 1.31 (Yeh *et al.,* 1999). The value of H' was measured at two levels: the average diversity within population (H_{POP}) and the total diversity (H_{SP}), than the proportion of diversity among populations was estimated as (H_{SP} - H_{POP})/ H_{SP}. An unweighted Pair-Group Method Arithmatic (UPGMA) dendogram analysis based on Jaccard's genetic distance was constructed using NTSYS version 2.00 (Rohlf, 1998) software.

PCR amplification products from 10 primers selected generated 26 DNA fragments. The band number per primer ranged 1-4 and band sizes ranged from 190-1190 bp (Table 1), depending on primer type used and sampled genotype. Amplification products generated by OPD-08, OPO-16, OPO-13 and OPT-07 primers displayed

Table 1. Primers selected and their sequences, size range of amplified fragments of *E. longifolia,* and total number of bands

Primer name	Sequence of the primers	Fragment size (bp)	Total number of bands
OPD-08	5'GTGCCCCATG'3	450-700	3
OPO-11	5'GACAGGAGGT'3	420-500	2
OPO-13	5'GTCAGAGTCC'3	760-1.190	3
OPO-16	5'TCGGCGGTTC'3	400-800	4
OPT-07	5'GGCAGGCTGT'3	560-1000	4
OPY-15	5'AGTCGCCCTT'3	350-550	3
OPY-16	5'GGGCCAATGT'3	450	1
OPY-17	5'GACGTGGTGA'3	810	1
OPY-19	5'TGAGGGTCCC'3	440	1
OPY-20	5'AGCCGTGGAA'3	190-475	4
Total	–	–	26

polymorphic patterns (Figure 1), while amplification products generated by OPY-20, OPO-11, OPY-15, OPY-19, OPY-16, and OPY-17 primers showed monomorphic patterns (Figure 2). Based on RAPD analysis in this study, we concluded that male and female *E. longifolia* did differ genetically because none of the primers amplified RAPD bands that were entirely absent in one of the type *E. longifolia* but present in the others. Furthermore, we suggested that large scale screening of random primers was necessary to develop specific markers and should be confirmed either by repeating the PCR reaction or by sequencing. These results also explained that difference in name of plants by local people based on morphological characters is not necessarily reflected in genetic differences. Many studies have

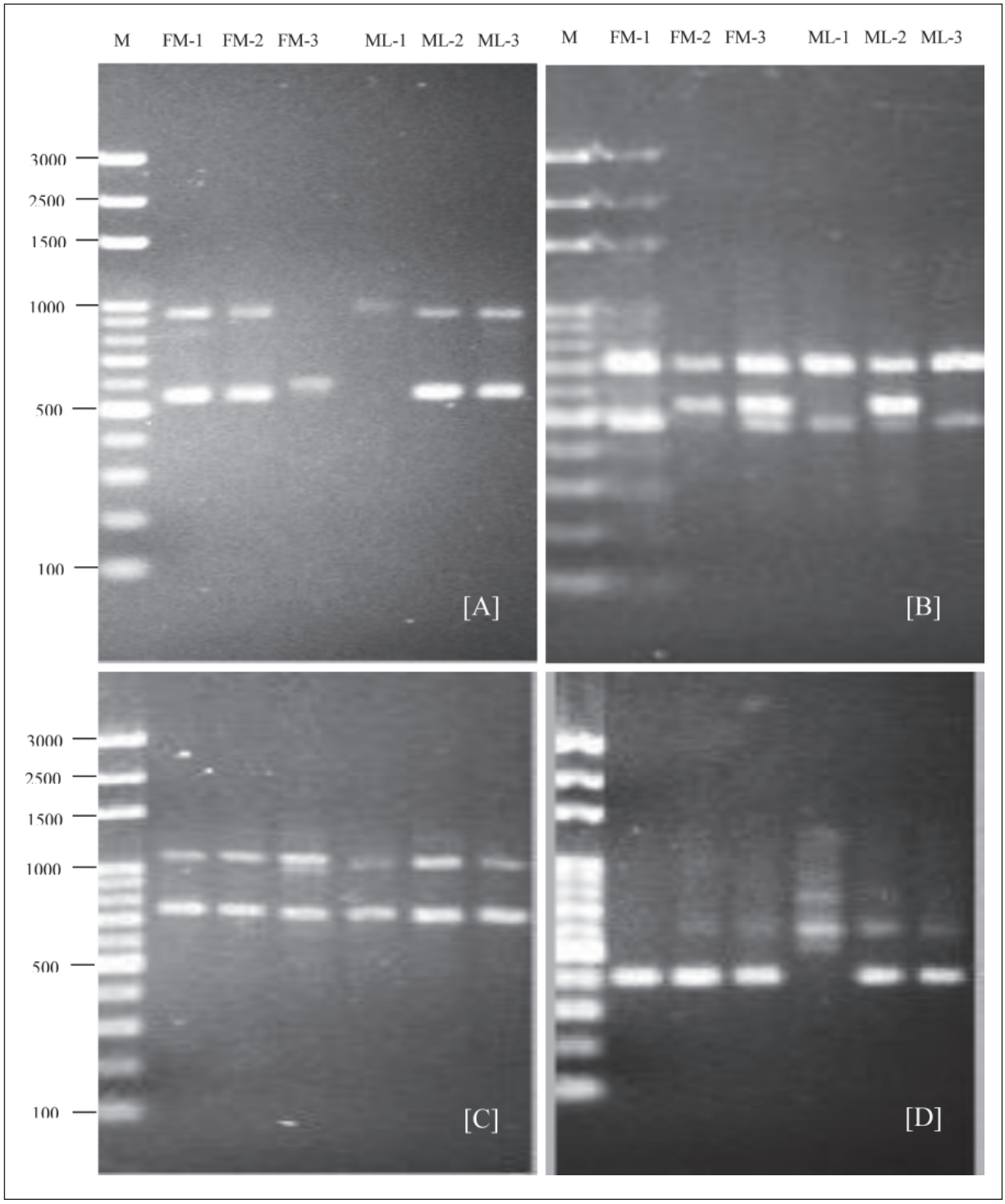

Fig. 1. RAPD amplification products by primers: OPT-07 [A]; OPD-08 [B]; OPO-13 [C] and OPO-16 [D]. FM-1-FM-3 [female *E. longifolia*] and ML-1-ML-3 [male *E. longifolia*].

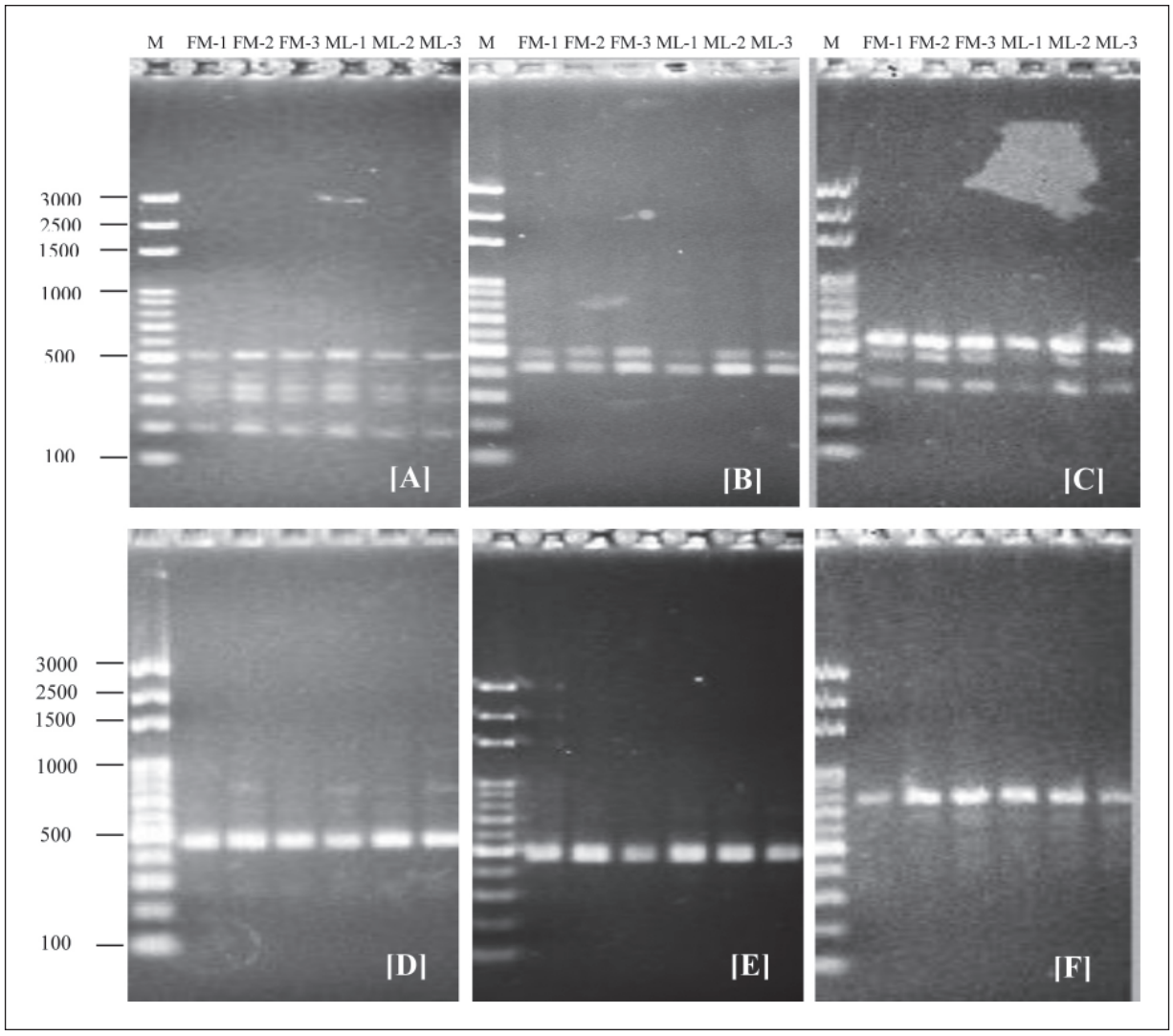

Fig. 2. RAPD amplification products by primers: OPY-20 [A]; OPO-11[B]; OPY-15[C]; OPY-19 [D]; OPY-16 [E]; and OPY-17[F]; FM-1-FM-3 [female *E. longifolia*] and ML-1-ML-3 [male *E. longifolia*].

Table 2. Genetic diversity indices of *E. longifolia*

Sampled	PPL (%)	H' (SE)
Female	23.08	0.1415 (0.2676)
Male	26.92	0.1519 (0.2608)
Average	25.00	0.1467 (0.2642)
Specias level	38.46	0.1812 (0.2564)

Note: PPL = percentage of polymorphic loci; H' = Shannon's diversity index.

been also reported that the species are different morphologically distinct but they are genetically indistinguishable and that often become debating among geneticist and taxonomist (Eurling & Gravendeel, 2005).

The percentage of polymorphic loci ranged from 23.08% for female *E. longifolia* to 26.92% for male *E. longifolia.* Polymorphic loci indicate that the genetic variation among male *E. longifolia* was higher than female *E. longifolia*. In the present study, Shannon's diversity index (H') estimated type of *E. longifolia* indicated that female *E. longifolia* had lower genetic variation than male *E. longifolia* (Table 2). Declining genetic variation in female *E. longifolia* might be attributed to the over collection of the plants in the Forest Reserve of Kenegerian Rumbio due to the fact that this type of *E. longifolia* is more bitter and local people claim that female *E. longifolia* is more effective than the male counterpart.

At the species level, the percentage of polymorphic loci and Shannon's diversity index was 38.46% and was 0.1812, respectively. The mean percentage of polymorphic loci in *E. longifolia* was lower than that reported for other long lived perennial plant species (48.1%) reported by

Hamrick and Godt (1996). Low polymorphic loci could be explained by small sample size (six individuals). The number of individuals sampled will determine the number of alleles captured in a population. Brown (1989) explained that the fraction of alleles captured increases in direct proportion to the natural logarithm of the sample size. This means that sampling 10% of individuals in a population would theoretically capture 60-70% of the alleles present, with decreasing additional amounts of allelic diversity detected with more individuals sampled.

The genetic diversity of *E. longifolia* in this study is lower than mean value of genetic diversity of *E. longifolia* (He = 0.31, Susilowati, 2009) and (He = 0.20, Rosmaina and Zulfahmi, 2013) using RAPD markers. The low genetic diversity occurred in *E. longifolia* in this study is not unexpected and could be attributed to several factors. First, the low percentage of polymorphic loci value and small number of sampled will cause low genetic diversity. According to Ward and Jasieniuk (2009) that genetic diversity was sensitive to both the number of individuals and the number of loci sampled. Second, small population sizes of *E. longifolia*. Our field investigations indicate that population sizes of *E. longifolia* are small, with few individuals in the population study. In addition, the habitats of *E. longifolia* are within secondary forests that have fragmented due to extended of rubber plantation and housing development by local people. Theoretically, reductions in population size cause genetic bottlenecks which tend to lower the proportion of polymorphic loci and reduce the number of alleles per locus within fragmented habitat, and lead to further loss of genetic diversity (Ellstand and Elam 1993, Young *et al.,* 1996). Moreover, pollination failure is very common in small and fragmented populations since pollinators are less attracted to the small sized populations (Andrieu *et al.,* 2009), that can consequently result in decreased fruit or seed set.

The value of genetic differentiation among populations $(H_{SP} - H_{POP})/H_{SP}$ estimated with Shannon's diversity index was 0.1904. This value indicated that 80.96% of the total genetic variation was found within population, whereas 19.04% of the variation found among populations. Several factors such as breeding system, gene flow and seed dispersal model effect population structure of *E. longifolia*. The value of genetic differentiation among female and male *E. longifolia* in this study was similar to average of genetic differentiation (G_{ST} = 0.19) for plant with mixed breeding system (Nybom and Bartish, 2000), and genetic differentiation for insect pollinated species (G_{ST} = 0.197) (David *et al.,* 1996). Thus, these values offer an indication of the possible reproductive system of *E. longifolia* was partial outcrossing and/or selfing which are pollinated by insects. This is supported by observation in green house of flower morphology in *E. longifolia* that male *E. longifolia* flowers possess pistil and stamen, but a sterile pistil, while the female *E. longifolia* possess pistil and stamen, but a sterile stamens. Based on floral morphology of *E. longifolia* above, we conclude that *E. longifolia* is a cross pollinated plant. Hamrick and Godt (1996) noted that cross pollinating plant species tend to have high levels of genetic variation within populations, but low levels of genetic differentiation among populations, whereas self-pollinating or asexually reproducing species typically have less within population genetic variation and more differentiation among populations.

High genetic differentiation value among species indicated that gene flow is restricted via pollen or seed. Seed of *E. longifolia* is relatively heavy and big so seed dispersal is limited on forest floor, most seeds were found close to parent trees (Keng *et al.,* 2002; Osman *et al.,* 2005). Hence, high genetic differentiation among species could be also explained by the low density of *E. longifolia* within the sampling area which would influence the mating system and gene flow among *E. longifolia*. The flowering tree density and performance of pollinator are probably responsible for low mating. The spacing of individuals within populations may affect the visitation rates and behavior of pollinators, and consequently, increasing levels of self-fertilization and gene flow distances. When the density of reproductive trees is reduced, both the distance among reproductive trees and the distance of pollen dispersal increase. The increase in the distance among reproductive trees forces the pollinator to travel longer distances between trees to obtain nectar, increasing the distance of pollen dispersal. Fukue *et al.* (2007) stated that long distance gene flow occurs more frequently in populations with a relatively low mature tree density. Alteration in population density can also affect the species self-incompatibility system, changing the mating system from predominantly outcrossing to a mixed mating system as has been reported for *Paquira quinata* (Fuchs *et al*., 2003) and *Ceiba pentandra* (Lobo *et al.,* 2005). Declining outcrossing rates and increasing in selfing rates were observed in low density populations of *Shorea curtisii* (Obayashi *et al.,* 2002) and of *Neobalanocarpus heimii* (Naito *et al.,* 2005). However, pollen flow may be particularly sensitive to density changes (Degen *et al.,* 2004; Jones and Hubbell, 2006). For example, increasing density has been found to decrease pollen flow distances in tropical tree species (Degen *et al.,* 2004), and where populations are fragmented or occur at low density,

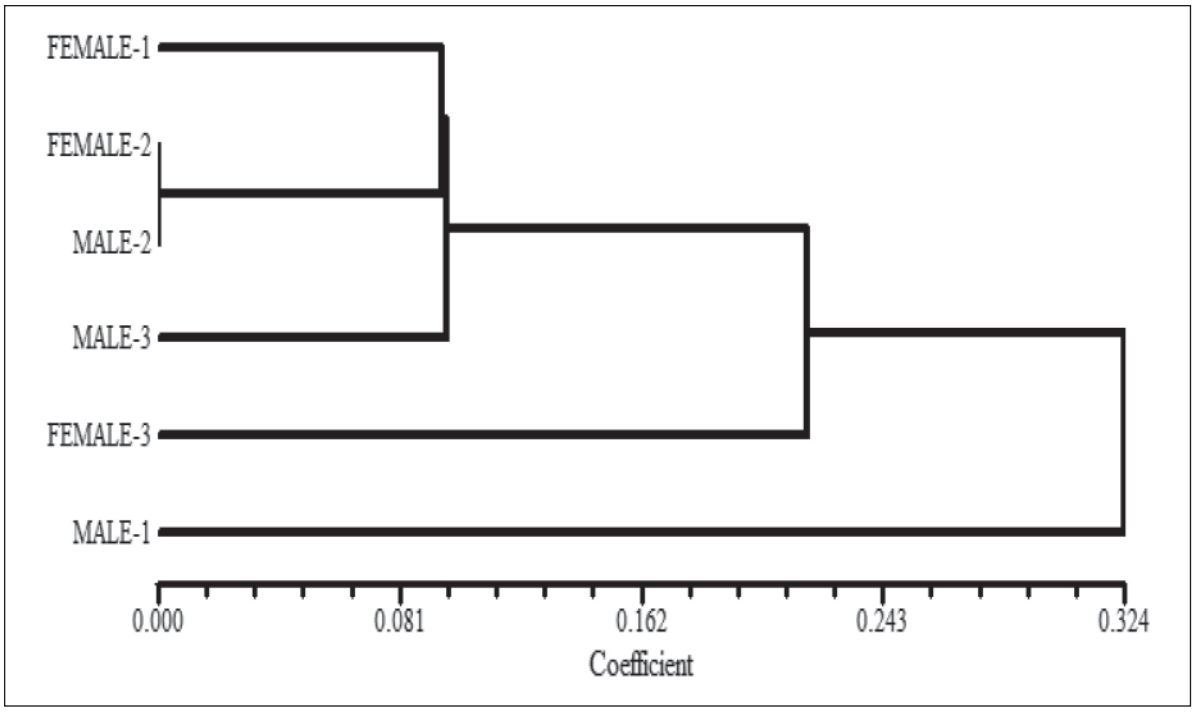

Fig. 3. Dendogram of UPGMA cluster analysis on female and male *E. longifolia* based on Jaccard's genetic distance.

pollen flow distances may increase (Dick *et al.,* 2008; Jha and Dick, 2010).

Estimation of gene flow (*Nm*) between female and male *E. longifolia* in our study was 2.117 individual per generation and belong to moderate category (Govindaraju, 1989). This value was higher than gene flow of *E. longifolia* that was reported by Rosmaina and Zulfahmi (2013) (*Nm* = 1.11) and gene flow values of tropical trees (*Nm* > 1.0, Lacerda *et al.,* 2001). The high gene flow (Nm > 1) of *E. longifolia* in this study is strong enough to counteract genetic drift (Slatkin, 1987; Slatkin and Barton, 1989). *Nm* > 1 in outcrossing species might be due to well developed pollen dispersal mechanisms of the *E. longifolia* plants. But, in the present study, genetic differentiation was also found to be high in the *E. longifolia*. The reason might be fragmented populations of a small size and all of them were under human influence, which is likely to lead to reductions of the population size or population isolation and extensive, recurrent gene flow. Gene flow is determined by pollinators, seed dispersers, stand density, flowers phenology, gender distribution, outcrossing rates and inbreeding depression (Dick *et al.,* 2008).

The Jaccard's genetic distance values between female and male of *E. longifolia* was ranged from 0.00 to 0.348. UPGMA dendogram based on Jaccard's genetic distance displayed that *E. longifolia* was divided into three groups (Figure 3). This dendogram did not clearly separated between female and male *E. longifolia* because no primers specific that can distinguish of them.

Knowledge of genetic diversity of species is crucial for conservation purposes. Considering the high level of genetic differentiation between female and male *E. longifolia* and low genetic variation in populations, both species should be considered for conservation. Population protection and pending of collection or harvesting of species required efforts to propagate *E. longifolia* through seed or alternative methods for the production of *E. longifolia* seedling required to be implemented and seedling produced should subsequently be reintroduced to forest reserve of Kenegerian Rumbio through enrichment planting to increase genetic diversity in natural habitats.

ACKNOWLEDGEMENTS

The authors thank Mr. Ripani and Manager of Forest Reserve of Kenegerian Rumbio for assistance during sample collection. We also thank two anonymous reviewers for their valuable comments.

REFERENCES

Andrieu, E., Dornier, A., Rouifed, S., Schatz, B. & Cheptou, P.O. 2009. The town crepis and the country crepis: how does fragmentation affect a plant pollinator interaction? *Acta Oecologia*, **35**: 1-7.

Chan, K.L., Choo, C.Y. & Abdullah, N.R. 2005. Semisynthetic 15-O-acyl- and 1,15-di-Oacyleurycomanones from *Eurycoma longifolia* as potential antimalarials. *Planta Medica*, **71**: 967-9.

David, B., MacDonald, C. & Hamrick, J.L. 1996. Genetic variation in some plants of Florida scrub. *American Journal of Botany*, **83**: 21-27.

Dick, C.W., Hardy, O.J., Jones, F.A. & Petit, R.J. 2008. Spatial Scales of Pollen and Seed-Mediated Gene Flow in Tropical Rain Forest Trees. *Tropical Plant Biology,* **1**: 20-33.

Dawson, I.K., Simons, A.J., Waugh, R. & Powell, W. 1995. Diversity and genetic differentiation among subpopulations of *Gliricidia sepium* revealed by PCR-based assays. *Heredity,* **74**: 10-18.

Degen, B., Bandou, E. & Caron, H. 2004. Limited pollen dispersal and biparental inbreeding in Symphonia globulifera in French Guiana. *Heredity,* **93**: 585-591.

Doyle, J.J. & Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus,* **12**: 13-15.

Effendy, N.M., Mohamed, N., Muhammad, N., Mohamad, I.N. & Shuid, A.N. 2012. *Eurycoma longifolia*: Medicinal Plant in the Prevention and Treatment of Male Osteoporosis due to Androgen Deficiency. *Evidence-Based Complementary and Alternative Medicine*. doi:10.1155/2012/125761.

Ellstrand, N.C. & Elam, D.R. 1993. Population genetic consequences of small population size: implications for plant conservation. *Annual Review Ecology and Systematic*, **24**: 217-242.

Eurling, M.C.M. & Gravendeel, B. 2005. TrnL-TrnF sequence data imply paraphyly of Aquilaria and Gyrinops (Thymelaeceae) and provide new perspectives for agarwood identification. *Plant Systematic and Evolution*, **254**: 1-12.

Fuchs, E.J., Lobo, J.A. & Quesada, M. 2003. Effects of forest fragmentation and flowering phenology on the reproductive success andmating patterns of the tropical dry forest tree *Pachira quinata*. *Conservation Biology*, **17**: 149-157.

Fukue, Y., Kado, T., Lee, S.L., Ng, K.K.S., Muhammad, N. & Tsumura, Y. 2007. Effects of flowering tree density on the mating system and gene flow in Shorea leprosula (Dipterocarpaceae) in Peninsular Malaysia. *Journal of Plant Research,* **120**: 413-420.

Govindaraju, R.D. 1989. Variation in gene flow levels among predominantly self-pollinated plants. *Journal of Evolution Biology,* **2**: 173-181.

Hadiah, J.T. 2000. *Eurycoma longifolia* Jack (Pasak Bumi). *Eksplorasi,* **2**: 6.

Hamrick, J.L. & Godt, M.J.W. 1996. Effects of life history trait on genetic diversityin plant species. *Philosophical Transactions Royal Society London Series Biological Sciences*, **351**: 1291-1298.

Jha, S. & Dick, C.W. 2010. Native bees mediate long-distance pollen dispersal in a shade coffee landscape mosaic. *Proceedings of the National Academy of Sciences United State of America,* **107**: 13760-13764.

Jones, F.A. & Hubbell, S.P. 2006. Demographic spatial structure of the neotropical tree, Jacaranda copaia. *Molecular Ecology*, **15**: 3205-3217.

Keng, C.L., Sai, S.T. & Teo, C.K.H. 2002. A preliminary study on the germination of *Eurycoma longifolia* Jack (Tongkat Ali) seeds. *Pertanika Journal of Tropical Agriculture Science,* **25**: 27-34.

Lacerda, D.R., Acedo, M.D.P., Filho, J.P.L. & Lovato, M.B. 2001. Genetic diversity and structure of natural populations of *Plathymenia reticulate* (Mimosoideae), a tropical tree from the Brazilian Cerrado. *Molecular Ecology,* **10**: 1143-1152.

Lewontin, R.C. 1972. The apportionment of human diversity. *Evolution Biology*, **6**: 381-398.

Lina, E.C., Arneti, Prijono, D. & Dadang. 2009. Insecticidal Potency of Pasak Bumi (*Eurycoma longifolia* Jack) against Cabbage Pest Crocidolomia pavonana (F.) (Lepidoptera: Crambidae). *Indonesian Journal of Entomology,* **6**: 21-29.

Lobo, J.A., Quesada, M. & Stoner, K.E. 2005. Effects of pollination by bats on the mating system of *Ceiba pentandra* (Bombacaceae) populations in two tropical life zones in Costa Rica. *American Journal of Botany*, **92**: 370-376.

Lynch, M. & Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. *Molecular Ecology,* **3**: 91-99.

Muchugi, A., Kadu, C., Kindt, R., Kipruto, H., Lemurt, S., Olale, K., Nyadoi, P., Dawson, I. & Jamnadass, R. 2008. Molecular Markers for Tropical Trees, A Practical Guide to Principles and Procedures. ICRAF Technical Manual No. 9. I, Dawson & R. Jamnadass (Eds.) Nairobi: World Agroforestry Centre.

Naito, Y., Konuma, A., Iwata, H., Suyama, Y., Seiwa, K., Okuda, T., Lee, S.L., Norwati, M. & Tsumura, Y. 2005. Selfing and inbreeding depression in seeds and seedlings of Neobalanocarpus heimii

(Dipterocarpaceae). *Journal of Plant Research*, **118**: 423-430.

Nybom, H. & Bartish, I.V. 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspectives in Plant Ecology, Evolution and Systematics*, 93-114.

Obayashi, K., Tsumura, Y., Ihara-Ujino, T., Niiyama, K., Tanouchi, H., Suyama, Y., Washitani, I., Lee, C.T., Lee, S.L. & Muhammad, N. 2002. Genetic diversity and outcrossing rate between undisturbed and selectively logged forests of *Shorea curtisii* (Dipterocarpaceae) using microsatellite DNA analysis. *International Journal of Plant Science,* **163**: 151-158.

Osman, A., Jordan, B., Lessard, P.A., Muhammad, N., Haron, M.R., Riffin, N.M., Sinskey, A.J., Rha, C.K. & Housman, D.E. 2005. Genetic Diversity of *Eurycoma longifolia* Inferred from Single Nucleotide Polymorphisms. *Plant Physiology,* **131**: 1294-1301.

Rohlf, F.J. 1998. NTSYSpc: Numerical Taxonomy. Department of Ecology and Evolution, State University of New York, Stony Brook.

Rosmaina and Zulfahmi. 2013. Genetic Diversity of *Eurycoma longifolia* Jack Based on Random Amplified Polymorphic DNA Marker. *Journal of Tropical Forest Management*, **XIX(2)**: 138-144, DOI: 10.7226/jtfm.19.2.138

Slatkin, M. 1987. Gene flow and the geographic structure of natural populations. *Science*, **236**: 787-792.

Slatkin, M. & Barton, N.H. 1989. A comparison of three indirect methods for estimating the average level of gene flow. *Evolution*, **43**: 1349-1368.

Susilowati, A. 2009. Teknik Perbanyakan dan Analisis Kekerabatan Pasak Bumi (*Eurycoma longifolia* Jack). Tesis. Sekolah Pascasarjana IPB. Bogor.

Tambi, M.I., Imran, M.K. & Henkel, R.R. 2011. Standardised water-solubleextract of *Eurycoma longifolia*, Tongkat ali, as testosterone booster for men with late- onset hypogonadism. *Andrologia,* **44**: 226-230.

Tee, T.T. & Azimahtol, H.L.P. 2005. Induction of Apoptosis by *Eurycoma longifolia* Jack Extracts. *Anticancer Research,* **25**: 2205-2214.

Ward, S.M. & Jasieniuk, M. 2009. Review: sampling weedy and invasive plant populations for genetic diversity analysis. *Weed Science*, **57**: 593-602.

Weising, K., Nybom, H., Wolf, K. & Kahl, G. 2005. DNA fingerprinting in plants: principles, methods, and applications. CRC Press, Boca Raton.

Welsh, J. & McClelland. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Research,* **18**: 7213-7218.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primer are useful as genetic markers. *Nucleic Acids Research,* **18**: 6531-6535.

Yeh, F.C., Yang, R. & Boyle, T. 1999. POPGEN Version 1.31. Microsoft Window based for population genetic analysis. Department Renewable Resources, University of Alberta, Edmonton, Alberta, Canada.

Young, A.G., Boyle, T. & Brown, T. 1996. The population genetic consequences of habitat fragmentation for plants. *Trends Ecology and Evolution*, **11**: 413-419.