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DIFFERENTIATION OF TWO SPECIES OF PASAK BUMI (*EURYCOMA* SPP) BASED ON LEAF MORPHOMETRIC

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Abstract

Forest Reserve of Kenegerian Rumbio, Riau-Indoensia was harbored two species of Pasak Bumi (Eurycoma spp) which is locally popular as male Eurycoma and female Eurycoma. The objective of this research was to investigate the leaf morphometric variation and dissimilarities betweenmale and female Eurycoma. Fifteen of leaf morphometric characters were measured in each species of Eurycoma. Analysis of variance,discriminant analysis (DA), principal component analysis (PCA), and cluster analysiswere performed. The results of analysis of variance and Duncan multiple range test showed that all parameters were significant differences. It revealed thatall of these variable could be utilized to differentiated male Eurycoma and female Eurycoma. The results of DA clearly defined the distinctiveness of the female and male Eurycoma that reflected from high percentages of correctly classified sample.The PCA was resolved into two principal components (PCs), which explained 44.83% and 35.33% of total variation. Scatter plot and UPGMA dendogram divided both eurycoma species into two groups, first group consisted of individuals from female eurycoma and second group consisted of individuals from male eurycoma. These results indicated that male eurycoma and female eurycoma were different species. Furthermore, the result identification of herbarium of both species inBogor Botanical Garden-Indonesia is also concludedthat they are different species, which male eurycoma was *Eurycoma longifolia* Jack, and female eurycoma was *Eurycoma apiculata*, A.W. Been. This finding could be usedto identify the appropriate species for utilization in the pharmaceutical, conservation and breeding program in future. Wealso recommended that comprehensive studies involving molecular analyses and phytochemical tests should be performed on the two species of Eurycoma.

Key words : Variation, leaf morphology, *Eurycoma*, Simaroubaceae.

Introduction

Eurycoma spp is an important medicinal plant which belongs to family Simaraubaceae. In Indonesia, this plant is only found in Borneo and Sumatra forests and itis locally known as PasakBumi. Recent research reported that the root extract of Eurycoma spp consist of various bioactive constituents including quassinoid, eurycomanol, eurycomanone, etc. (Bhat and Karim, 2010; Abubakar *et al*., 2017) and its compounds areused to antileukaemic (Morita *et al*., 1993), antimalaria, anticancer, aphrodisiac, antitumor (Rashid *et al*., 2009; Abubakar *et al*., 2017), antimicrobial (Khanam *et al*., 2015) and toxicity effect (Razak *et al*., 2011).

Nowadays, *Eurycoma* spp has been threatened due to the decline in the number of individuals, habitats fragmentation and isolation, lack of natural regeneration and disturbance of natural population structure as impact of human activities. Therefore, conservation of Eurycoma through in-situ and ex-situ methods is a priority to be implemented and knowledge of its genetic diversity is required for conservation programs. Genetic diversity plays a crucial role in the plant adaptationto their natural environments and to cope with the stresses encountered due to changes inthe environment including biotic and abiotic (Alfaro *et al.,* 2014). In addition, natural selection

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can take place only when sufficient genetic diversity (Hughes *et al*., 2008). In the otherhand, baseline data on the naturally accuring morphological variability is one of the requirment for establishing defined species that will be needed to produce medicinal product with homogenous quality and establishing the species conservation.

Forest reserve of Kenegerian Rumbio was harbored two species of eurycoma,which they is locally known asmale eurycoma and female eurycoma. According to local society that the naming of these species did not relate to their reproduction system and theydifferentiated both species through the color of petiole and level of bitter. A question is appearing, whether both species were similar or different? To answear this question, as a initial step, we conducted the leaf morphometric analysis of both species. We chose leaves to be analysed because leaves were easy to be obtained and measuredand have a strong strength to discriminate between plant species. In addition, the leaf morphometric information of Eurycoma is absent. Leaf morphometric analysis is a valuable tool in genetic studies, taxonomy, biogeography and species evolution (Bayramzadeh *et al*., 2012). Cope *et al*. (2012) stated that leaf characteristics have been frequently used by botanists to identify species, such as to distinguish Quercus species (Kremer *et al*., 2002; Boratynski *et al*., 2008; Uslu and Bakiş, 2014), *Actaea racemosa* (Gardener *et al*., 2012), *Monstera adansonii* (Mayo and Andrade, 2013), two african mahagonies (Danquah *et al*., 2011).

The natural stands of Eurycoma are the main source of germplasm in plant breeding for the production of medicines and conservation program so that validity the species taxonomy or identifying appropriate species become important before its activities to be implemented. The objective of the present study was to assess the leaf morphomectric variation of male Eurycoma and female Eurycoma and to know whether male eurycoma and female Eurycoma were similar or different species. This information can be used to identify the appropriate species in designing a framework for conservation projects and pharmaceutical development in Indonesia in future.

Materials and Methods

The study was carried out on two species of Eurycoma (male and female Eurycoma) thatis found in Forest Reserve of Kenegerian Rumbio, Riau-Indonesia. A total of 121 adult trees were sampled that consisted of 32 and 89 plants for male eurycoma and female eurycoma, respectively. The differences number of sample collected is caused by restricted availability of individuals in studyarea. To prevent sampling adult trees from the same parent or pedigree, a minimum distance of 20-50m on average was maintained between each tree. Each tree was taken one leaf that fully developed and free of insect and disease attack to be measured. After sampling, the leaves were herbarised in Breeding and Genetic Laboratory, Faculty of Agriculture and Animal Science of State Islamic University of Sultan Syarif Kasim (UIN SUSKA) Riau, then herbarium is sent to Bogor Botanical Garden to identified of these species. The parameter measured included leaf and leaflet characters were as shown in table 1. Two of these traits were ratios or synthetic variables, meant to capture leaf and leaflet shapes. All characters were measured using digical caliper and number of leaflet were counted.

Table 1 : List of leaf morphological traits examined.

No.	Abbreviation	Morphological trait
1.	LL (cm)	Leaf length
2.	LD (cm)	Leaf width
3.	PL (cm)	Petiole length
4.	LLW (cm)	Distance from base to point of maximal width
5.	NL	Number of leaflet
6.	PD (cm)	Petiole Diameter of leaf
7.	RL (cm)	Rachis length
8.	LL/LD	Ratio of Leaf length to leaf width
9.	LFW2 (cm)	Leaflet width at 1 cm beneath the leaft apex
10.	LFW3 (cm)	Leaflet width from the central vein to the left leaf margin, measured at half of the leaflet length.
11.	LFW4 (cm)	Leaflet width from the central vein to the right leaf margin, measured at half of the leaflet length.
12.	LFW 5 (cm)	Leaflet width at 0.5 cm above the leaflet base
13.	LFL (cm)	Leaflet length
14.	LFW1	Leaflet width, measured at half of the laeflet length (LFW1= LFW3+LFW4)
15.	LFL/LFW1	Ratio of leaflet length to leaflet width

Data analysis

The parameters of descritive statistics were calculated for morphological traits of Eurycoma spp *viz* mean and standar deviation (SD). Analysis of variance (ANOVA) was performed to all characters and significant differences were determined at $p<0.01$. All characters that significant different were conducted of further test of duncan multiple range test. The similarity and dissimilarity between species of each sample in a species was determined using discriminant analysis and classification analysis of all measured data werealso performed. Principle component analysis (PCA) on fifteen characters were calculated to determine the contribution of each variable to the separation of the species. Cluster analysis using the hierarchical unweighted pair group method with arithmetic averages (UPGMA) was performed based on matrix of euclidean distance. ANOVA was conducted using Statistical Analysis System (SAS) version 9.1; discriminant analysis and PCA were performed using XLSTAT software package (www.xlstat.com) while UPGMA was performed using MVSP version 3.22 Software (www.kovcomp.com).

Results and Discussion

Morphometric variation analysis

Analysis of variance showed the significant differences for all the quantitative traits. This indicated that sufficient amount of variation was present between species for all quantitative traits. Duncan multiple range test showed significant differences between averages of two species for all observation parameters (table 2), it displayed that all of these variables can be used to distinguish male and female Eurycoma. The mean and standard deviations of all morphological characters of the two species of Eurycoma can be observed in table 2. Male eurycoma have the highest mean value for leaf length (LL), leaf width (LD), petiole length (PL), distance from base to point of maximal width (LLW), petiole diameter (PD), number of leaflet (NL), rachis length (RL), ratioof leaf length to leaf width (LL/LD), leaflet length (LFL) and ratioleaflet length to leaflet width (LFL/LFWI) whereas female eurycoma have the highest mean value on leaflet width at 1 cm beneath the leaft apex (LFW2), Leaflet width from the central vein to the left leaf margin (LFW3), Leaflet width from the central vein to the right leaf margin (LFW4), Leaflet width at 0.5 cm above the leaflet base (LFW5) and the leaflet width (LFW1).

Leaf length mean was 45.77 cm for male eurycoma and 30.08 cm for female eurycoma while leaf diameter was 15.56 cm for male eurycoma and 14.07 cm for female eurycoma. Average of petiole length was 10.04 cm and 8.24 cm for male eurycoma and female eurycoma, respectively. Zarafshar *et al*. (2010) stated that petiole length is strongly associated with environmental conditions. Average of leaf length/width ratio of male eurycoma was 2.91 and average of leaf length/width ratio of female eurycoma was 2.16. Average of leaflet length/width ratio was 3.89 for male eurycoma and 2.84 for female eurycoma. Leaflet length/width reflected leaflet shape. Chen and Nelson (2004) defined five categories for leaflet shape based on length/width ratio value: less than 2.0 (oval); 2.1 to 3.0 (ovate); 3.1 to 4.0 (lanceolate); 4.1 to 5.0 (linear) and over 5.0 (ultra linear). Based on above classified that leaf shape of male eurycoma is lanceolate and leaflet shape of female eurycoma was ovate.Leaflet shape eurycoma spp in this study a line with to leaf shape eurycoma (ovate-lanceolate) that is explained by Nooteboom (1962). Mean of leaflet length of male eurycoma and female eurycoma was 7,63 cm and 6.80 cm, respectively. According to Chen and Nelson (2004) that leaflet length is divided into three categories, namely small leaflet is less than 6.0 cm in length; intermediate leaflet from 6.1 to 10.0 cm and large leaflet over 10.0 cm. Therefore, both male and female eurycoma having leaflet length was intermediate category.

The high variation between individuals of Eurycoma is part of phenotypic flexibility because not only the variation in the micro-environment condition of the growing site experienced by each plant but also the result of genetic differences among the Eurycoma individuals concerned. The high diversity can also be caused by the fact that there is no intensive breeding or selection. The Eurycoma plants at the study area are only generatively generated through the seeds and produce the extent of variation found within individuals in the species as impact of genetic recombination due to the crossing-over process between male and female gametes at the time of miosis. In addition, Hubert and Cottrell (2007) stated that genetic variation of a given species present at a site was determined by several factors as natural selection, genetic drif, gene flow and mutation. Bruschi *et al*. (2003) suggested that variability in observational intra and inter-species morphological features canprovide a basis for the improvement and storage of genetic diversity of plant.

Differentiation of two species Eurycoma

Discriminant function analysis was conducted on the entire set of 121 individuals. The final discriminant function analysis produced highly significant (Wilks Lambda = 0.359; F statistic = 13.519; $P<0.0001$). The discriminant analysis clearly defined the distinctiveness of the two

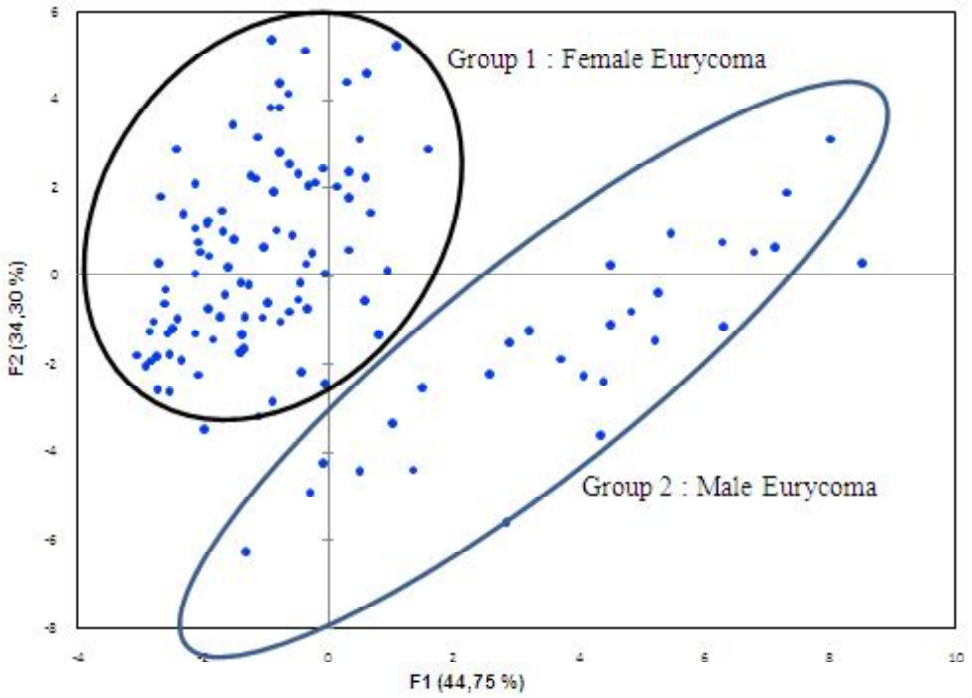

Fig. 1 : Scatterplot of the principle component analysis for male and female eurycoma is along of first and second principle components.

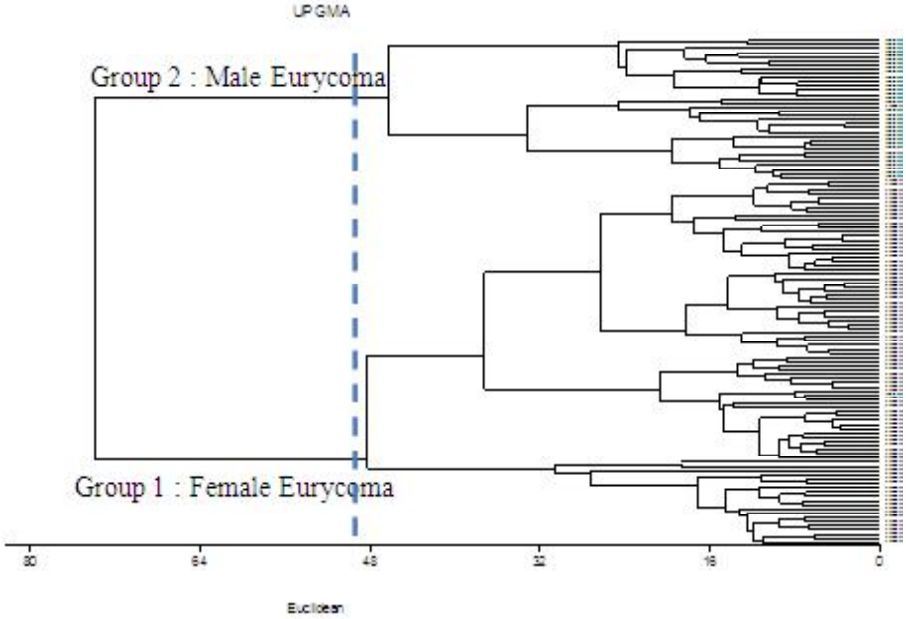

Fig. 2 : UPGMA dendogram of 121 individuals Eurycoma in forest reserve of Kenegerian Rumbio, Riau.

species (female and male of eurycoma). All data measured were classified into groups to which they most likely belonged (table 3). Based on the fifteen variables, the samples of female Eurycoma were correctly classified in 95.40% of the cases. Those samples could be misclassified as samples of the male eurycoma in 4.60% of the cases. The samples of male eurycoma were correctly classified in 82.35% of the cases and there were 17.65% misclassified as samples of female eurycoma. Generally, results of the discriminant analysis classification showed a high percentage of correctly classified cases to two species (in total 91.73%, table 3). The misclassified of individual was probably occured because there are individuals hybrids between male eurycoma and female eurycoma. Natural hybrid between male and female eurycoma may also occur because both species of eurycoma lived in the same habitat, both species have similar the flowering time (July-august) and the pollination systems of both plants are cross-pollinated. Several studies have also reported the presence of natural hybrids

Table 2 : Descriptive statistical parameters and result of ANOVA of the each variable.

Variable	Parameters	Eurycoma		Results of ANOVA	
		Male	Female	F-hit	Pr> F
LL	$\bar{x}$ ± SD (cm)	45.77a±1.79	30.08b±1.12	55.42	<0.000
LD	$\bar{x}$ ± SD (cm)	15.56a±0.51	14.07b±0.32	6.12	<0.015
PL	$\bar{x}$ ± SD (cm)	10.04a±0.52	8.24b±0.33	8.57	<0.004
LLW	$\bar{x}$ ±SD (cm)	33.69a±1.40	22.31b±0.88	47.59	<0.000
NL	$\bar{x}$ ±SD (cm)	32.38a±0.95	19.02b±0.60	140.93	<0.000
PD	$\bar{x}$ ±SD (cm)	2.15a±0.08	1.61b±0.05	35.23	<0.000
RL	$\bar{x}$ ±SD (cm)	35.84a±1.44	21.48b±0.90	71.34	<0.000
LL/LD	$\bar{x}$ ±SD (cm)	2.91a±0.09	2.16b±0.06	52.52	<0.000
LFW2	$\bar{x}$ ± SD (cm)	1.34b±0.07	1.68a±0.04	18.23	<0.000
LFW3	$\bar{x}$ ± SD (cm)	0.95b±0.04	1.20a±0.02	36.25	<0.000
LFW4	$\bar{x}$ ± SD (cm)	1.03b±0.04	1.23a±0.0.02	19.82	<0.000
LFW5	$\bar{x}$ ± SD (cm)	1.12b± 0.73	1.65a± 0.04	40.98	<0.000
LFL	$\bar{x}$ ± SD (cm)	7.63a±0.24	6.80b±0.15	9.05	<0.003
LFW1	$\bar{x}$ ± SD (cm)	1.98b±0.08	2.43a± 0.04	28.43	<0.000
LFL/LFW1	$\bar{x}$ ± SD (cm)	3.89a±0.10	2.84b±0.06	85.69	<0.000

Table 3 : Classification matric of two species of Eurycoma with cases correctly classified (%).

Eurycoma	Female	Male	Correctly classified
Female	95.40	4.60	95.40
Male	17.65	82.35	82.35
Total			91.73

Table 4 : Eigen values, proportional and cumulative per cent variation and principalcomponent for 14 traits.

	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	6.73	5.30	0.85	0.56	0.45	0.31
Variability (%)	44.83	35.34	5.70	3.72	2.98	2.04
Cumulative %	44.83	80.17	85.87	89.59	92.57	94.61

of closely related plants such as natural hybrid of *Shorea leprosula* x *Shorea parvifolia*, *Shorea leprosula* x *Shorea* curtisii (Kamiya *et al*., 2011), *Quercus* (Kremer *et al*., 2002; Barozan and Babac, 2003; Gonzales-Rodriques and Oyama, 2005; Ito, 2009), Ceanothus (Rhamnaceae) (Hardig *et al*., 2002) and *Carex flava* (Wieclaw and Wilhelm, 2014). To prove the existence of natural hybrid between the two species eurycoma is required further research.

The results of principal component analysis (PCA) can be observed in fig. 1, which produced 14 eigen values for 14 eigen vectors but we discussed only six eigen vectors. The six principal components (PC1-PC6) explained more than 94% of the total variance, and the rest of the component (PC7-PC14) varied to a lesser extent (with total 6%) (table 4). The first two principal components accounted for approximately 80% of the variation. The characters which gave the highest contribution to firstprincipal component were leaf length (LL), distance from base to point of maximal width (LLW), number of leaflets (NL), petiole diameter of leaf (PD) and rachis length (RL), which explained 44.86% of the total variation. This is indicated by high positive loadings of eigen vectors of the first principal component (PC1). The high factor loadings in second principal component wererelated toleaflet width at 1 cm beneath the leaf apex (LFW2), Leaflet width from the central vein to the left leaf margin (LFW3), leaflet width from the central vein to the right leaf margin (LFW4), leaflet width at 0.5 cm above the leaflet base (LFW5) and the leaflet width (LFW1) which accounted for 35.34% of the variation.

The third principal component (PC3) was related to Ratio of Leaf length to leaf width (LL/LD), leaflet length (LFL), Ratio of leaflet length to leaflet width (LFL/LFW1) and leaf width (LD) while the traits with highest factor loadings in PC4, PC5 and PC6 were petiole length (PL), leaflet width at 1 cm beneath the leaf apex (LFW2) and distance from base to point of maximal width (LLW), respectively (table 5). All the principal

Table 5 : Estimates of the weighting coefficient (Eigen vector) associated with the principal components and different characters of eurycoma.

Characters	PC1	PC2	PC3	PC4	PC5	PC6
LL	**0.378**	0.006	0.141	-0.002	-0.038	0.004
LD	0.270	0.235	**-0.347**	0.198	0.071	0.402
PL	0.269	0.102	0.076	**0.841**	-0.289	-0.157
LLW	**0.340**	0.010	0.052	-0.165	-0.064	**0.620**
NL	**0.329**	-0.169	0.117	-0.018	0.103	0.066
PD	**0.332**	0.052	0.150	-0.264	0.115	-0.183
RL	**0.374**	-0.020	0.100	-0.075	0.000	0.046
LL/LD	0.287	-0.159	**0.510**	-0.123	-0.083	-0.331
LFW2	-0.012	**0.349**	0.273	0.137	**0.717**	0.121
LFW3	0.000	**0.416**	-0.010	-0.147	-0.269	-0.080
LFW4	0.061	**0.411**	-0.024	-0.174	-0.186	-0.072
LFW5	-0.068	**0.383**	0.134	0.113	0.307	-0.229
LFL	0.299	0.157	**-0.481**	-0.156	0.064	-0.324
LFW1	0.032	**0.422**	-0.018	-0.164	-0.232	-0.077
LFL/LFW1	0.238	-0.252	**-0.468**	0.024	0.319	-0.301

Characters with high loadings on each of the principal components are indicated in bold

components caused a dispersion of the individuals along a continuos gradient with a distinct cluster. The projection of the first two principal components is shown in fig. 1. The PCA ordination plots depicted a clearly separation between male eurycoma and female eurycoma.

The UPGMA dendogram are displayed in fig. 2. The result of UPGMA dendogram was also confirmed result of PCA that divided both eurycoma species into two main groups, first group consisted of individuals from female eurycoma and second group consisted of individuals from male eurycoma. This results indicated that male eurycoma and female eurycoma were different species. Based on field observation on leaf or leaflet of two species of eurycoma and comparing the results of this study to characteristic of eurycoma species that is described by Nooteboom (1962), we estimated that male eurycoma was *Eurycomalongifolia* while female eurycoma was *Eurycoma apiculata*. To validate our estimation, the herbarium samples of the both species are sent to the Bogor Botanical Garden, Indoensia for further identification purposes. The result of the identification of the herbarium in Bogor Botanical Garden stated that the male eurycoma is *Eurycoma longifolia*, Jack and the female eurycoma is *Eurycoma apiculata*, A.W. Benn. This result is very surprising due to eurycoma species popular in Indonesia only species of *Eurycoma longifolia*, while *Eurycoma apiculata* has not yet been reported and the results of our study found the type *Eurycoma apiculata*. This is the first reportthat *Eurycoma apiculata* existed in Sumatra -Indonesia. This is supported by Nooteboom (1962), which states that the deployment of the *Eurycoma apiculata* is not only in the Malaysian peninsula but also in Sumatra.

Based on results of this study are concluded that leafmorphomectic traits can easily distinguish two eurycoma genotypes, male eurycoma and female eurycoma were different species which scientific name of male eurycoma was *Eurycoma longifolia* Jack and female eurycoma was *Eurycoma apiculata*, A.W. Been.

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