

# PHENETIC AND PHYLOGENETIC ANALYSES OF TENKAWANG (*Shorea* spp., DIPTEROCARPACEAE) BASED ON MORPHOLOGICAL AND MOLECULAR DATA

## Analisis fenetik dan filogenetik Tengkawang (*Shorea* spp., Dipterocarpaceae) berdasarkan data morfologi dan molekuler

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### Abstrak

Tengkawang merupakan kelompok tumbuhan yang terdiri atas sekitar 16 jenis *Shorea* yang menghasilkan minyak *illipe* dari bijinya. Nama Tengkawang seringkali merujuk pada *Bornean Illipe Nut*, walaupun tidak keseluruhan jenis-jenis Tengkawang tersebar di pulau Borneo. Sembilan dari ke-16 jenis tersebut merupakan anggota dari *Shorea* seksi *Pachycarpae*, dua jenis dari seksi *Brachypterae*, tiga jenis dari seksi *Mutica* dan dua jenis dari seksi *Shorea*. Hasil dari studi filogenetika molekuler yang telah dilakukan belakangan ini menunjukkan bahwa seksi *Pachycarpae* cenderung membentuk group yang monofiletik. Penelitian ini bertujuan untuk mempelajari pola pengelompokan Tengkawang dan kekerabatannya berdasarkan karakter morfologi dan data molekuler. Analisis fenetik dan kladistik dilakukan pada tiga belas jenis *Shorea*. Dendrogram UPGMA Tengkawang dibuat berdasarkan persentase koefisien kemiripan. Seluruh jenis tengkawang membentuk satu kelompok berdasarkan 70% koefisien kemiripan. Relatif tingginya koefisien kemiripan (70%) yang dijumpai pada 13 jenis tengkawang ini selaras dengan pengelompokan berdasarkan jenis kayu. Seluruh jenis *Shorea* seksi *Pachycarpae* pada penelitian ini memiliki koefisien kemiripan sebesar 78%, sedangkan hasil analisis *Principal Component Analysis* (PCA) menunjukkan posisi 13 jenis Tengkawang dalam dua dimensi. Berdasarkan hasil analisis PCA, seluruh 35 karakter morfologi yang digunakan mendefinisikan *Shorea* seksi *Pachycarpae* kedalam satu group. Analisis filogenetika berdasarkan beberapa gen pada genom chloroplast juga dilakukan sebagai pembandingan, hasil dari analisis filogenetika ini mirip dengan hasil analisis fenetik, dimana *Shorea* section *Pachycarpae* membentuk kelompok monofiletik yang terletak di terminal kladogram.

**Kata kunci:** Tengkawang, analisis fenetik, filogenetik, kelompok

### Abstract

Tengkawang is referred to a group of approximately 16 species of *Shorea* producing illipe butter from their kernels. The name Tengkawang mostly referred to the Bornean Illipe Nut, even though they are not restrictly distributed in Borneo. Nine of the 16 species belonging to *Shorea* Section *Pachycarpae*, two species of Section *Brachypterae*, three species of Section *Mutica* and two species of Section *Shorea*. Recent phylogenetic studies

based on molecular markers considered Section *Pachycarpae* tend to form a monophyletic group. This present study was aimed to investigate the nature of groupings within Tengkawang and their relatedness based on phenotypic traits and molecular data. Thirteen species of *Shorea* were selected to perform a phenetic and cladistics analyses. An UPGMA dendrogram of Tengkawang was obtained using percentage similarity coefficient. All species tengkawang were united in a group at similarity coefficient of 70%. The relatively high similarity (70%) that shared among 13 species of tengkawang has supported the timber grouping of tengkawang. *Shorea* section *Pachycarpae* shared 78% similarity among them. The principal Component Analysis (PCA) was also performed and showed the relative position of 13 of Tengkawang in two-dimension. The PCA has also identified all the 35 morphological traits having important roles in the grouping of *Shorea* section *Pachycarpae*. A comparative phylogenetic analysis using several chloroplast genes was also in accordance to the results of phenetic analysis, and that the Bornean endemic *Shorea* section *Pachycarpae* was a monophyletic group located at the terminal cladogram.

**Keywords:** Tengkawang, phenetic analysis, lipe nut, group

## INTRODUCTION

Tengkawang is a group of *Shorea* (Dipterocarpaceae, Meranti-merantian) consisted of ~16 species (Table 1). This group was considered a variable group belonging to Red Meranti/Light hardwood (13 species), and Balau/Heavy hardwood (3 species) that distributed in Peninsular Malaysia, Sumatra dan Borneo. Seven species of Red Meranti were belonging to *Shorea* section *Pachycarpae*, endemic to Kalimantan (Ashton, 1982) and some of them have been successfully planted in other islands, Jawa and Sumatra, such as *S. pinanga*, *S. stenoptera* and *S. macrophylla*.

Amongst 16 species of tengkawang recognised, *Shorea stenoptera* (Tengkawang tungkul) was the most utilised since it has the largest fruit size. Other species that have been harvested widely were *S. pinanga* and *S. macrophylla* because they are more widely distributed. Analysis of fatty acid of tengkawang seeds have also been carried out on *S. macrophylla*, *S. seminis* dan *S. palembanica* (Anderson, 1975). Extract of fatty acid from tengkawang seed have similar characteristics to those of cocoa seeds, hence tengkawang fat have often been used as substitute of cocoa fat in food, cosmetic and medicinal industries. In the international market, fat extracted from tengkawang was known as *borneo tallow* or *green butter* (Winarni *et al.*, 2004). The bornean tallow was used as an

alternative raw ingredient of cocoa fat was termed *cocoa butter equivalent* (CBE), *cocoa butter substitutes* (CBS) and *cocoa butter replacers* (CBR) (Blicher-Mathiesen, 1994).

In the perspective of systematic study, however, it was interesting to address the issue on how the tengkawang species were grouped when these 16 species were belonging to different sections of *Shorea* but shared similar fatty acid. Morphological, anatomical and molecular characters were often used by systematists to evaluate classification or grouping of certain taxa. Study on phylogenetic relationship of tengkawang has not been done exclusively but there were some previous studies on molecular phylogeny of Dipterocarpaceae using several genes/regions within chloroplast and nuclear (Dayanandan *et al.*, 1999; Kamiya *et al.*, 2005; Yulita *et al.*, 2005; Gamage *et al.*, 2006; Tsumura *et al.*, 2011; Yulita, 2013; Yulita, 2001).

*Shorea* are variable genera consisting the largest number of species within the Dipterocarps family. The variability of morphological characters mostly due to many overlapping and continuous characters that make it difficult to delineate the species. Hence, most taxonomists avoided the use of morphological characters to perform analysis related to classification of Dipterocarps. This present study was aimed to investigate the nature of groupings within Tengkawang and their relatedness based on

phenotypic traits. Results from this study will be an important information to be used - for instance- to select potential species that have similar features possessed by the most utilized tengkawang *i.e.* *S. stenoptera* that can be further developed.

## MATERIALS AND METHODS

### Morphology

Thirteen species of *Shorea* from 65 herbarium specimens were selected for a phenetic analysis (Table 2).

Thirty five morphological characters comprised 23 qualitative and 12 quantitative

characters of leaves, fruits, individual flower and part of floral organs (Table 3). The continuous quantitative characters were coded numerically following Thiele (1993).

The complete data set (Tabel 3) was analysed using UPGMA (Unweighted Pair Group Method with Arithmetic Average) cluster analysis (Sneath and Sokal, 1973) and ordination using PCA (Principal Component Analysis). Cluster analysis using coefficient of percentage similarity with equal weight linkage method (UPGMA) and PCA were performed using MVSP (Multi Variate Statistical Package, Kovach 2007).

**Table 1.** List of Tengkawang species (compiled from Ashton, 1982).

No	Scientific name	Section	Vernacular name	Timber group	Distribution
1	<i>Shorea amplexicaulis</i> P.S.Ashton <sup>a</sup>	<i>Pachycarpae</i>	tengkawang mege	Red Meranti (Light hardwood)	West Kalimantan
2	<i>Shorea beccariana</i> Burck <sup>a</sup>	<i>Pachycarpae</i>	tengkawang tengkal	Red Meranti (Light hardwood)	West Kalimantan
3	<i>Shorea fallax</i> Meijer <sup>c</sup>	<i>Brachypterae</i>	engkabang layar	Red Meranti (Light hardwood)	Kalimantan
4	<i>Shorea havilandii</i> Brandis <sup>b</sup>	<i>Shorea</i>	selangan batu pinang, tengkawang ayer	Balau (Heavy hardwood)	Kalimantan
5	<i>Shorea lepidota</i> (Korth.) Blume <sup>d</sup>	<i>Mutica</i>	tengkawang gunung	Red Meranti(Light hardwood)	Sumatra
6	<i>Shorea macrantha</i> Brandis <sup>d</sup>	<i>Mutica</i>	engkabang bungkus	Red Meranti (Light hardwood)	Sumatra, Kalimantan
7	<i>Shorea macrophylla</i> (de Vriese) P.S.Ashton <sup>a</sup>	<i>Pachycarpae</i>	tengkawang hantelok	Meranti merah	West Kalimantan
8	<i>Shorea mecystopteryx</i> Ridl <sup>a</sup>	<i>Pachycarpae</i>	tengkawang layar	Red Meranti (Light hardwood)	West Kalimantan
9	<i>Shorea palembanica</i> Miq. <sup>c</sup>	<i>Brachypterae</i>	tengkawang majau	Red Meranti (Light hardwood)	Sumatra, Kalimantan
10	<i>Shorea pinanga</i> Scheff. <sup>a</sup>	<i>Pachycarpae</i>	tengkawang rambai	Red Meranti (Light hardwood)	West Kalimantan
11	<i>Shorea scaberrima</i> Burck <sup>c</sup>	<i>Brachypterae</i>	tengkawang kijang	Red Meranti (Light hardwood)	Kalimantan
12	<i>Shorea seminis</i> (de Viese) v.Slooten <sup>b</sup>	<i>Shorea</i>	tengkawang terendak	Balau (Heavy hardwood)	Kalimantan
13	<i>Shorea singkawang</i> (Miq.) Miq. <sup>d</sup>	<i>Mutica</i>	sengkawang pinang	Red Meranti (Light hardwood)	Sumatra
14	<i>Shorea splendida</i> (de Vriese) P.S.Ashton <sup>a</sup>	<i>Pachycarpae</i>	tengkawang bani	Red Meranti (Light hardwood)	West Kalimantan
15	<i>Shorea stenoptera</i> Burck <sup>a</sup>	<i>Pachycarpae</i>	tengkawang tungkul	Red Meranti (Light hardwood)	West Kalimantan
16	<i>Shorea sumatrana</i> Sym. ex Desch <sup>b</sup>	<i>Shorea</i>	kedawang, tengkawang batu	Balau (Heavy hardwood)	Sumatra, Kalimantan

**Table 2.** Selected taxa for analyses of phenetic and cladistics

Species	Abbreviation	Herbarium collection number (for phenetic analysis)	Genbank accession number
<i>Anisoptera laevis</i>	ALAEV	-	AB452617, AB452409, AB452201
<b><i>Shorea</i> section <i>Shorea</i></b>			
<i>S. seminis</i>	SSEMI	A 578, A 367, 7934, Uthman Ismani S.37049, S. 15576, SAN 21211	AB452774, AB452566, AY026633
<i>S. sumatrana</i>	SSUM	-	AB452798, AB452590, AB452382
<b><i>Shorea</i> Section <i>Brachypterae</i> subsection <i>Smithiana</i></b>			
<i>S. smithiana</i>	SSMIT	SAN 21483, bb.19.773, bb.19.985, AA.1164A, SAN-134957, bb.32.556, K. Sidayasa 435	-
<b><i>Shorea</i> Section <i>Brachypterae</i> Subsection <i>Brachypterae</i></b>			
<i>S. palembanica</i>	SPALE	A.51B, A 1459, TH Erdert 742	AB452733, AB452525, AY026624
<b><i>Shorea</i> Section <i>Pachycarpae</i></b>			
<i>S. amplexicaulis</i>	SAMPL	Jarvie 5756, Jarvie 5331, S 29174, S 46406	AB452622, AB452621, AY026603
<i>S. rotundifolia</i>	SROTU	29207, S-29539, S-29207, S-29226, S 30001	-
<i>S. macrophylla</i>	SMACR	TL 1073, TL 1125, BO-71908, BO- 117008	AB452707, AB452706, AY026618
<i>S. pinanga</i>	SPING	TL 1194, bb. 30.190, bb. 29. 636, BO- 74699, BO-74830, bb. 20.021	AB452755, AB452547, AY026628
<i>S. splendida</i>	SSPLE	Jurie 5270, bb.29.674, bb. 31.436, Kepong 98877	AB452791, AB452583, AY026636
<i>S. stenoptera</i>	SSTEN	BO-0114891, BO-0114889, bb. 29.664, bb. 29.289, Peters.1036, L8- 533	AB452797, AB452588, AY026637
<i>S. pilosa</i>	SPILO	22380, 23970, bb. 30.208, SAN 97229, S.22380	AB452754, AB452546, AY026627
<i>S. beccariana</i>	SBECC	S 46468, SAR 29174, S. 46468, Ambra & Arifin, Berau 1088, S. 29243	AB452630, AB452422, AY026605
<b><i>Shorea</i> Section <i>Mutica</i> subsection <i>Mutica</i></b>			
<i>S. singkawang</i>	SSING	FRI 21684, FRI 25407, 85280, 76638, 6504, 99359, Leg in BO1269256, 3083	AB452777, AB452569, AY026634

## Molecular

Twelve species of *Shorea* (Table 2) were also selected and an out group (*Anisoptera laevis*). DNA sequences of *psbC*, *psbC-trnS* intergenic spacer, tRNA-His, *psbA*, *psbA-trnK* intergenic spacer, *trnL-trnF* intergenic spacer, tRNA-Phe were obtained from the genbank NCBI (Table 2).

Cladistic analysis using Maximum Likelihood was also performed using Mega 6.06 (Tamura *et al.*, 2013). Data analysis was set as Tamura Nei nucleotide substitution model, Gamma distributed (G), ML heuristic method using Nearest Neighbour Interchange (NNI), and 100 bootstrap replication.

**Table 3.** List of characters and character states used in this study.

No	Name of character	States, and number of multistate for quantitative characters
1	Stipule scars	0=absent, 1=present, 2=0&1
2	Length of leaf lamina	0-11
3	Width of leaf lamina	0-11
4	Tertiary leaf nervation	0=scalariform, 1= reticulate
5	Domatia	0=absent, 1=present, 2=0&1
6	Length of individual flower bud	0-11
7	Width of individual flower bud	0-10
8	Persistence of bract	0=caduceus, 1=persistent
9	Persistence of bracteoles	0=caduceus, 1=persistent, 2=0&1
10	Presence of hairs on sepal surface	0=absent, 1=present, 2=0&1
11	Presence of hairs on petal surface	0=absent, 1=present
12	Length of petal	0-11
13	Number of stamens	0=>15, 1=up to 15
14	Number of rows of stamens	0=> two rows, 1=up to two rows
15	Type of anther connective appendage	0=broad appendage widest in the upper part, 1=semi broad (an intermediate form, between 0 and 2), 2=filiform
16	Length of anther connective appendage	0-11
17	Presence of hairs on anther connective	0=absent, 1=present
18	Length of anthers	0-11
19	Width of anthers	0-10
20	Presence of hairs on anthers	0=absent, 1=present
21	Presence of stellate hairs on filament	0=absent, 1=present
22	Presence of neck in filament	0=absent, 1=present, 2=0&1
23	Length of filament	0-9
24	Presence of stylopodium	0=absent, 1=present, 2=0&1
25	Length of pistil	0-10
26	Presence of hairs on the ovary	0=absent, 1=present, 2 = 0&1
27	Length of style	0-10
28	Presence of hairs on style	0=absent, 1=present
29	Presence of fruit pedicel	0=absent, 1=present, 2=0&1
30	Comparative development of fruit calyx	0=subequal/equal, 1=2 long and 3 short sepals, 2=3 long and 2 short sepals
31	Shape of fruit wings	0=spatulate, linear, 1=triangular, 2=rounded
32	Length of nut	0-11
33	Width of nut	0-11
34	Presence of hairs on nut	0=absent, 1=present, 2=0&1
35	Presence of style remnant	0=absent, 1=present, glabrous, 2=present, not glabrous

## RESULTS AND DISCUSSION

### Phenetic analysis using morphological data

Based on UPGMA dendrogram, all species tengkawang were united in a group at similarity coefficient of 70% (A, Figure 1). The relatively high similarity (70%) that shared among 13 species of tengkawang has supported the timber grouping of

tengkawang. *Shorea seminis* and *S. palembanica* (section *Brachypterae*) were separated from the other species and was located at the basal dendrogram. The other species were forming two clusters (A and B, Figure 1). Subcluster A comprised *Shorea macrantha* and *S. singkawang* (Section *Mutica*) united at 82% similarity, while subcluster B consisted of all members of section *Pachycarpae*

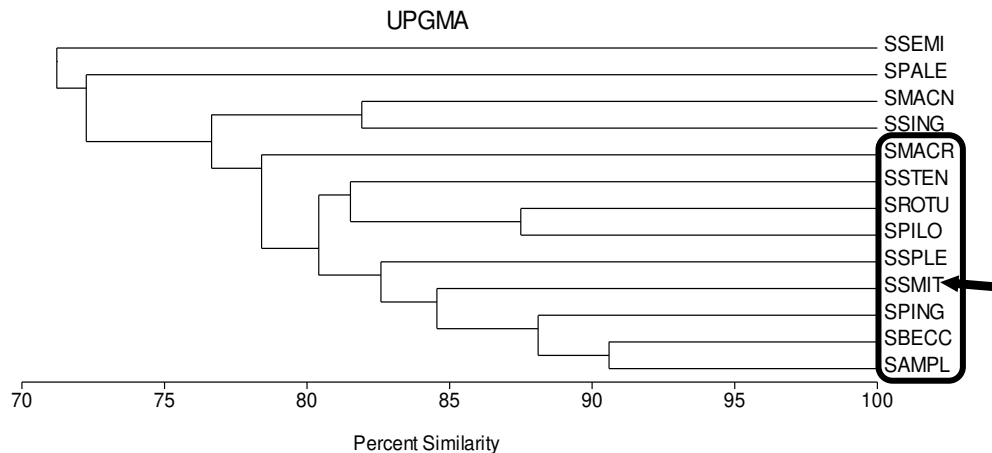
shared 78% similarity among them. Within section *Pachycarpae*, *Shorea beccariana* and *S. amplexicaulis* were the most similar species by having 92% similarity. The difference between these two subclusters were very low, only 4%, this may suggested the existence of overlapping characters defined the two subclusters.

Observation from the herbarium specimens and field collections showed that species of section *Pachycarpae* has comparatively larger fruit size than species from other section. Results from PCA suggested that all the 35 morphological characters used in this analysis were positively supported section *Pachycarpae* as a group (Figure 2). High similarities on morphological characters observed in this section may have resulted from long history of adaptation of this section to local habitat, climate, and pollinators. The fact that this section is naturally endemic to Borneo may have also suggested that they may have undergone adaptive radiation within this island. This may indicated that section *Pachycarpae* is indeed a distinct group of Tengkawang. Ashton (1982), however, defined this section based on similarities of filament, style and stylopodium. Some more comprehensive studies on molecular phylogeny of *Shorea* suggested that all species of Tengkawang were included within group of Red Meranti – a variable species consisted of *Shorea* section *Brachypterae*, *Mutica*, *Ovalis*, *Pachycarpae*

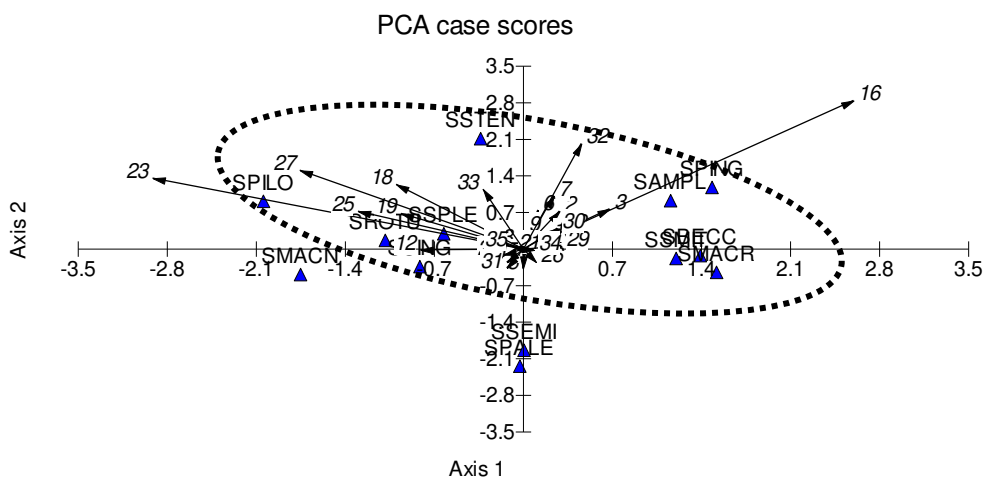
and *Rubella*. Within group of Red Meranti, most of species of section *Pachycarpae* tend to form a group together (Kamiya *et al.*, 2005; Yulita, 2001; Yulita *et al.*, 2005; Yulita, 2013; Indrioko *et al.*, 2006; Tsumura *et al.*, 2011).

*Shorea seminis*, a member of Balau group were placed at the basal of subcluster C, and this was in accordance to other molecular phylogenetic studies that separate Balau Group out of Red Meranti (Kamiya *et al.*, 2005; Tsumura *et al.*, 2011). In this study, *S. seminis* do not received any support from morphological characters (Figure 2). The interesting fact was that *S. seminis* that have the most distinct shape of fruit wings and relatively smaller fruit size, were extracted for Tengkawang illipe nut. This species was locally abundant, riparian species at low altitude on mainly clay soil. The nuts were collected and generally favoured for preparation for illipe butter (Anderson, 1975).

*Shorea palembanica* was also separated from other species. PCA diagram showed that this species was also not supported by any morphological character (Figure 2). The species has medium fruit size but have wider distribution since it occurred in east of Sumatra and Borneo but was locally abundant in clay rich alluvium soil (Anderson, 1975; Ashton, 1982).



**Figure 1.** UPGMA dendrogram of Tengkawang species based on coefficient of percent similarity. Taxa were abbreviated. Taxa in boxes are *Shorea* section *Pachycarpae* except for *Shorea smithiana* (section *Brachypterae*, pointed by an arrow)



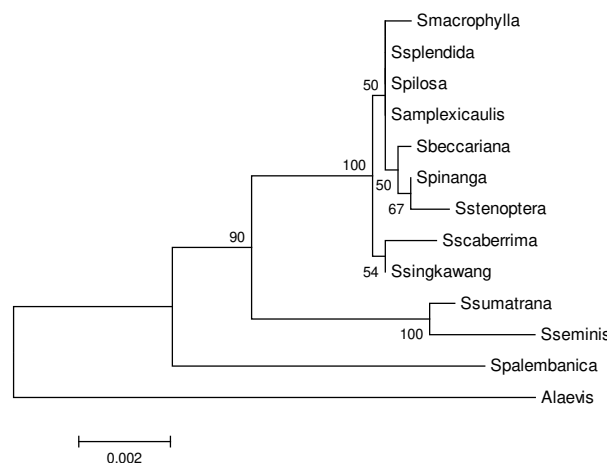
**Figure 2.** PCA diagram of Tengkadang species overlaying 35 morphological characters. Species in dotted circle are *Shorea* section *Pachycarpae*.

**Phylogenetic analysis using molecular data**

A comparative phylogenetic analysis of Tengkadang species was also performed using multigenes from chloroplast genomes (Figure 3). This analysis used *Anisoptera laevis* as an outgroup. Tengkadang species have been well supported as monophyletic group (90% bootstrap value). Section *Pachycarpae* form monophyletic group (100% bootstrap value) and sister to a clade united *S. scaberrima* and *S. singkawang*. These two clades was a Red Meranti group. Two species of Balau group, *S. sumatrana* and *S. seminis*, were united in different monophyletic clade with 100% support from bootstrap analysis. This result confirmed the monophyletic nature of section *Pachycarpae*. Meanwhile, *S. palembanica* was at the basal of Tengkadang clade suggesting that this species may have more ancient genomic properties than other species.

In the study of systematics, many scholars have avoided to use morphological characters because phenotypic traits is more prone to environment plasticity. Inclusion of morphological characters have often been limited due to many overlapping and continuous characters frequently

found in taxa with high variations such as *Shorea* species. Development of molecular techniques have permitted taxonomists to employ many region on chloroplast and nuclear genome as more accurate characters to perform cladistic (phylogenetic) and phenetic analysis. Results of this study showed that morphological data can be as reliable as molecular data. Selection of morphological characters can be an important stage before performing a phenetic analysis.



**Figure 3.** Cladogram of maximum likelihood of 12 species of Tengkadang and *Anisoptera laevis*. Taxa in box are species of section *Pachycarpae*. Numbers below branches are percentage bootstrap support.

## CONCLUSION

It is concluded that Tengawang species consisted of two groups, first was a group of section *Pachycarpae* and the second was remaining species belonging to other section/timber group. Section *Pachycarpae* may be a natural group defined by 35 morphological characters used in the analysis. Results from phenetic analysis using 35 morphological characters are almost in accordance to molecular phylogenetic analysis using multigenes from chloroplast genomes.

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