A TAXONOMIC STUDY ON RAFFLESIA TUAN-MUDAE
(RAFFLESIACEAE) IN SARAWAK

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This Final Year Project 2013 is based on my original work except for quotation and citation which have been acknowledged. I also declared that it has not been submitted previously or currently to any other degree at Universiti Malaysia Sarawak or any other institution.

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<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
<td></td>
</tr>
<tr>
<td>IUCN</td>
<td>International Union of Conservation of Nature</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>CIA</td>
<td>chloroform-isomylalcohol</td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl trimethylammonium bromide</td>
<td></td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
<td></td>
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<tr>
<td>ITS</td>
<td>Internal Transcribe Spacer</td>
<td></td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
<td></td>
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<tr>
<td>dntP</td>
<td>Deoxyribonucleotide triphosphate</td>
<td></td>
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<tr>
<td>MP</td>
<td>Maximum parsimony</td>
<td></td>
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<tr>
<td>ML</td>
<td>Maximum likelihood</td>
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<tr>
<td>LRT</td>
<td>Likelihood ratio test</td>
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<td>SFC</td>
<td>Sarawak Forestry Corporation</td>
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<tr>
<td>HKY</td>
<td>Hasegawa-Kishino-Yano</td>
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<tr>
<td>BIC</td>
<td>Bayesian Information Criterion</td>
<td></td>
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<tr>
<td>nrDNA</td>
<td>nuclear ribosomal DNA</td>
<td></td>
</tr>
<tr>
<td>CI</td>
<td>Consistency Index</td>
<td></td>
</tr>
<tr>
<td>RI</td>
<td>Retention Index</td>
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A Taxonomic Study on *Rafflesia tuan-mudae* (Rafflesiaceae) in Sarawak

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**ABSTRACT**

*Rafflesia tuan-mudae* is an endemic species of Sarawak. However, there is no complete description of *R. tuan-mudae*. Field work was done at Gunung Pueh, Gunung Gading and other localities such as Kg. Temurang, Kg. Begu and Lanjak Entimau to sample the blooming *R. tuan-mudae*. Detailed measurements and description of *R. tuan-mudae* were made. Voucher specimens of *R. tuan-mudae* from Gunung Pueh was preserved as wet specimen in spirit and deposited at Universiti Malaysia Sarawak Herbarium. Plant DNA materials of unknown *Rafflesia* spp. were taken from bracts or and perigone lobe and stored in silica gel. The DNA material was extracted, amplified and sequenced using nuclear ITS region. Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were carried out to estimate the phylogenetic tree to help in species identification. From the results obtained, it showed that the unknown *Rafflesia* spp. is closely related to *R. tuan-mudae* based on the Maximum Parsimony and Maximum Likelihood analyses with moderate bootstrap support. From the alignment obtained, it clearly showed that the sequence of the unknown *Rafflesia* spp. is similar to the known *R. tuan-mudae* from the GenBank except one mutation at site 787 that differentiate *R. tuan-mudae* with the unknown *Rafflesia* spp. Therefore, the unknown species could probably be *R. tuan-mudae*. However, more data are needed to confirm this result.

**Keyword:** *Rafflesia tuan-mudae*, taxonomy, Internal Transcribe Spacer

**ABSTRAK**


**Kata kunci:** *Rafflesia tuan-mudae*, taksonomi, Spaser Transkripsi Dalaman,
CHAPTER 1

INTRODUCTION

Tropical rainforest in Southeast Asia are confined to Borneo, Peninsular Malaysia, and Sumatra where it is hot and humid throughout the year (Kato, 2009). Malaysia comprised of tropical rainforest which have many unique floras and faunas. The ‘Queen of Parasite’, Rafflesia from the Rafflesiaceae family is one of the most unique and beautiful flowers ever known in botanical world (Nais, 2001) (Figure 1). It is also the largest flower in the world (Nais, 2001).

According to Nais (2001), Rafflesiaceae comprises eight genera and 55 species. Out of eight genera from Rafflesiaceae, there are three genera of bizzare endoholoparasitic flowering plants which are Rafflesia, Rhizanthes and Sapria (Nais, 2001). Rafflesia is exceedingly rare. There are about 20 species that have been recognized which mostly located in tropical rainforest of Southeast Asia (Nais, 2001) and occurs exclusively to the west of Wallace’s line (Bendiksby et al., 2010).

Rafflesia can be said as an icon of conservation, especially in the rainforest. Rafflesia is categorized as totally protected species in Sarawak (Thiessen, 2008). There are four species that can be found in Sarawak which are R. tuan-muda Beccari, R. hasseltii Suringar, R. keithii Meijer and R. pricei Meijer (Nais, 2001).
Problem statement and objectives

There are some Rafflesia species that is difficult to identify. There is morphological confusion between \textit{R. tuan-muda}e with \textit{R. arnoldii} and \textit{R. kethii}. Moreover, herbarium specimens are often in bad condition and no single herbarium has specimen of all species identified (Bendiksby \textit{et al.}, 2010). \textit{Rafflesia tuan-muda}e is an endemic species of Sarawak (Nais, 2001). However, there is no complete description of \textit{R. tuan-muda}e. This species was first collected from Mount Pueh, Sarawak, from which specimen of this species was recognized by Beccari in 1868. Unfortunately, in 1875, Beccari changed his mind and sunk it to a synonym of \textit{R. arnoldii}. Then, this species was revived by Solm-Laubach in 1891, based on dimension of the flower which is smaller than \textit{R. arnoldii} and have different pattern of warts on the perigone lobe (Nais, 2001).

Therefore, the objectives of this project are 1) to make a complete description of \textit{R. tuan-muda}e and have a voucher herbarium specimen, 2) to sequence the DNA of the unknown \textit{Rafflesia} from various localities and 3) to compare sequences from unknown \textit{Rafflesia} with published work to confirm the species.

Figure 1: Blooming flower of \textit{R. tuan-muda}e
2.1 Taxonomy, morphology and pollination of the genus *Rafflesia*

*Rafflesia* is one of the unique floras and is known as the largest flower of the world (Nais, 2001). According to Nais (2004), *Rafflesia* can achieve up to 107 cm in diameter as shown in *R. arnoldii*. *Rafflesia* is restricted to the western part of the phytogeographical region of Malesia, which is known as the Sunda shelf (Nais, 2001).

There are about 20 *Rafflesia* species that have been recognized and the list of species is shown in Table 1. There are eight species that can be found in Malaysia and five species are endemic to Malaysia (Nais, 2001). The species are *R. azlanii* and *R. cantleyi* in Peninsular Malaysia, *R. keithii* in Sabah and Sarawak, *R. tengku-adlinii* in Sabah and *R. tuan-mudae* in Sarawak (Nais, 2001).

*Rafflesia* is rootless, without chlorophyll, always rich in tannins and flowers usually unisexual (dioeciously) (Meijer, 1997) (Figure 2, Figure 3). *Rafflesia* is a holoparasitic plant which grow as strands of cells embedded within the stem and root tissues of their host (Bendiksby et al., 2010). Therefore, *Rafflesia* obtained their nutrients from their host plant. The host of *Rafflesia* is *Tetrastigma* (Vitaceae) which is a woody climber.
The main part of *Rafflesia* is the perigone which is a tube-like lower portion and it is reddish-brown in color. The top of the perigone tube is surrounded by five petal-like lobes and a shelf like structure called the diaphragm that protrudes towards the centre of the flower, producing an aperture in the middle (Mat Salleh, 1991). The inner wall of perigone tube is covered with numerous hair like-appendages called ramenta.

Table 1: *Rafflesia* in Southeast Asia (adapted from Nais, 2001)

<table>
<thead>
<tr>
<th>Species</th>
<th>Geographic origin</th>
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<tbody>
<tr>
<td>1) <em>R. arnoldii</em> Brown</td>
<td>Sumatra, Kalimantan</td>
</tr>
<tr>
<td>2) <em>R. gadutensis</em> Meijer</td>
<td>Sumatra</td>
</tr>
<tr>
<td>3) <em>R. hasseltii</em> Suringar</td>
<td>Sumatra, Sarawak</td>
</tr>
<tr>
<td>4) <em>R. micropylora</em> Meijer</td>
<td>Sumatra</td>
</tr>
<tr>
<td>5) <em>R. patma</em> Blume</td>
<td>Sumatra, Java</td>
</tr>
<tr>
<td>6) <em>R. rochussenii</em> Teijsmann &amp; Binnendijk</td>
<td>Sumatra, Java</td>
</tr>
<tr>
<td>7) <em>R. zollingeriana</em> Kooders</td>
<td>Java</td>
</tr>
<tr>
<td>8) <em>R. kerrii</em> Meijer</td>
<td>Thailand, Peninsular Malaysia</td>
</tr>
<tr>
<td>9) <em>R. azlanii</em> Latiff &amp; M. Wong</td>
<td>Peninsular Malaysia,</td>
</tr>
<tr>
<td>10) <em>R. cantleyi</em> Solms</td>
<td>Peninsular Malaysia</td>
</tr>
<tr>
<td>11) <em>R. keithii</em> Meijer</td>
<td>Sabah, Sarawak</td>
</tr>
<tr>
<td>12) <em>R. pricei</em> Meijer</td>
<td>Sabah, Sarawak</td>
</tr>
<tr>
<td>13) <em>R. tengku-adlinii</em> Mat-Salleh &amp; Latiff</td>
<td>Sabah</td>
</tr>
<tr>
<td>14) <em>R. tuan-mudae</em> Beccari</td>
<td>Sarawak</td>
</tr>
<tr>
<td>15) <em>R. borneensis</em> Kooders</td>
<td>Kalimantan</td>
</tr>
<tr>
<td>16) <em>R. ciliata</em> Kooders</td>
<td>Kalimantan</td>
</tr>
<tr>
<td>17) <em>R. witkampii</em> Kooders</td>
<td>Kalimantan</td>
</tr>
<tr>
<td>18) <em>R. manillana</em> Tescehemacher</td>
<td>Luzon, Samar, and Leyte (Philippines)</td>
</tr>
<tr>
<td>19) <em>R. schadenbergiana</em> Geoppert</td>
<td>Mindanao, Philippines</td>
</tr>
<tr>
<td>20) <em>R. specioca</em> Bercelona &amp; Fernando</td>
<td>Panay, Philippines</td>
</tr>
</tbody>
</table>
For classification of *Rafflesia* purposes, the shape and size of ramenta are important as they are consistent characters for a species (Mat Salleh, 1991). When *Rafflesia* is blooming, it will produce a stinky smell like rotten meat. *Rafflesia* has 5-6 petals that called perigone lobe. There are warts on the surface of perigone lobe. These warts are important for species identification.

![Diagram of female flower of Rafflesia](image2.png)

**Figure 2:** Female flower of *Rafflesia* (adapted from Nais, 2001)

![Diagram of male flower of Rafflesia](image3.png)

**Figure 3:** Male flower of *Rafflesia* (adapted from Nais, 2001)
The flowers of *Rafflesia* are thought to mimic rotting flesh and are 10-100 times larger in diameter than those of most flowering plant genera (Barkman et al., 2008). This unpleasant odor will attract pollinators. Mat Salleh (1991) stated that botanist John Beaman was the first person who studied and discussed about pollination of *Rafflesia*. Beaman et al. (1988) discovered that the smell of *Rafflesia* attracted flies, especially the bluebottle or carrion flies of the genera *Lucilia* and *Chrysomyia*, the main pollinators. The method of pollination needs male and female flowers bloom at about the same time in the same area (Mat Salleh, 1991).

The blooming of male and female *Rafflesia* flowers is related to its life cycle. There is a sequence of stages that involves in *Rafflesia*’s life cycle. *Rafflesia* needs 46 months to have a complete life cycle (Nais, 2004) (Figure 4). The germination of a seed marks the start of the present generation as follows: 1) germination of seeds; 2) development of the *Rafflesia* plant inside the host plant; 3) emergence of flower buds; 4) flowering; 5) pollination; 6) fruit and set seed; 7) dispersal of seeds; and 8) germination of seed which is the establishment of the next generation (Nais, 2001).
Figure 4: The life cycle of *Rafflesia*. (Adapted from Nais, 2001)
2.2 Phylogeny of *Rafflesia*

Barkman *et al.*, (2007) used a comparison of mitochondrial DNA (mtDNA) sequences to place *Rafflesia* in its phylogenetic context among the angiosperms. From their finding, it shows that *Rafflesia* is more closely related to *Rhizanthes* than to *Sapria*.

Barkman *et al.*, (2004) claimed that nuclear sequence data could provide an important independent genomic estimate of holoparasitic relationships as well. He stated that future studies of nuclear genes from *Rafflesia* and other extreme holoparasitic plants are needed because no other loci besides the 18S ribosomal DNA have been sequenced (Barkman *et al.*, 2004).

Therefore, in 2010, a phylogeny of *Rafflesia* based on mitochondrial genome (16S gene, matR and atp6 genes and the nad1 B-C intron) and the nuclear ribosomal ITS region was carried out. It shows that the family’s three component genera form a strongly supported monophyletic groups, and *Rafflesia* and *Rhizanthes* are sister clade (Bendiksby *et al.*, 2010). It also showed that Indonesian, Bornean, Peninsular Malaysia and the Philippines species of *Rafflesia* are reciprocally monophyletic, although the Bornean clade is not supported by bootstrapping above 50% (Bendiksby *et al.*, 2010).
2.3 *Rafflesia tuan-muda* Beccari

According to Nais (2004), *R. tuan-muda* endemically can be found in Sarawak. *Rafflesia tuan-muda* can be found in Mount Gading National Park at Lundu, Mount Pueh, and Mount Penrissen (Nais, 2001) (Figure 5). *Rafflesia tuan-muda* could probably be found in Mount Rara, however, this is not confirmed (Nais, 2001). According to Nais (2001), the first specimen was found in Mount Pueh, Sarawak and was named as *R. tuan-muda* by Beccari in 1868.

The species was dedicated to Mr. Carlo (Charles) Brooke Tuan Muda of Sarawak referring to the young future Rajah of Sarawak. Vernacular name of this species is ‘Pakma’ (Zuhud et al., 1998) as cited in Nais (2001). According to Beccari (1902) as cited in (Meijer, 1997), the habitat of *R. tuan-muda* is in lowland Dipterocarp forest.

According to Meijer (1997), *R. tuan-muda* have medium-sized flower which approximately 44-56 cm in diameter. The lower face of diaphragm and ramenta is much like *R. arnolidii* (Nais, 2001) but with perigone tubes only about 5-8 whitish warts across the broadest part (Meijer, 1997).

There is no information on pollination of *R. tuan-muda* yet. Therefore, a study on pollination of *R. tuan-muda* specifically and other *Rafflesia* species is needed.
2.4 Conservation status

In general, the status of *Rafflesia* species is poorly understood. So far, only *R. magnifica* is listed as critically endangered species under Red List of International Union of Conservation of Nature (IUCN) (2012). So, *Rafflesia* species need to be protected to avoid it from being extinct. There are five factors that can cause the species to extinct. The factors are: limited distribution and rarity of *Rafflesia*, large sex imbalance, specific host plants, high bud mortality rate and low level of pollination success and fruit set (Tan *et al.*, 2009). *Rafflesia* species are difficult to find due to its rarity and high mortality rate (Sofiyanti *et al.*, 2007).
The only way to conserve *Rafflesia* is to preserve its habitat (Mat Salleh, 1991). If the ecology and biodiversity have been destructed, probably the host will be lost and *Rafflesia* will have no host to live in (Mat Salleh, 1991). Tan *et al.*, (2009) stated that human activities such as collection of *Rafflesia* buds for traditional medicine, slash-and-burn agriculture and logging further threaten the population.

2.5 Ecotourism attraction

The uniqueness of this gigantic red-bloom of *Rafflesia* is one of the best attractions to promote ecotourism in Southeast Asia especially in Malaysia. According to Nais and Wilcock (1998) as cited by Tan *et al.*, (2009), one *Rafflesia* bloom could attract RM200 - RM8000 per annum if each *Rafflesia* site could produce 2 - 10 blossoms in a year. Therefore, it is important to conserve its habitat since it is important for ecotourism attraction such as The *Rafflesia* Conservation Incentive Scheme that was initiated in Sabah on 1994 (Mat Salleh *et al.*, 2006). It has allowed Indigenous landowners at Poring and Ranau on the edge of Kinabalu Park to earn visitor fees of 200 - 8000 ringgit annually from taking visitors to see flowering *Rafflesia* plants (Mat Salleh *et al.*, 2006).
CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

Field work was carried out to sample as many blooming *R. tuan muda*e as possible in Sarawak around Mount Gading National Park, Mount Pueh, Lanjak Entimau, Kampung Temurang and Kampung Begu Padawan. Samples from near Mount Gading National Park, Mount Pueh, Lanjak Entimau, Kampung Temurang, and Kampung Begu Padawan were collected by the SFC staff (Table 2). When flowers are present, detailed photographs were taken and detail measurements and observations were made. Then, the plant materials for DNA analysis were taken from perigone lobes and/or bracts of blooming *Rafflesia* and preserved in silica gel following protocol of Chase & Hills (1991). For phylogenetic analysis, *Rhizanthes lowii* was chosen as outgroup (Barkman et al., 2008).

3.2 Taxonomic description

Taxonomic description of *Rafflesia* was done following the description of Nais (2001). The measurements and description that were made are: 1) The diameter of aperture (cm); 2) the diameter of whole diaphragm (cm); 3) the diameter of open flower (cm); 4) the number and diameter of perigone lobe (cm); 5) the number of disk processes; 6) the number and patterns of warts on upper surface of diaphragm; 7) Colour and pattern of perigone lobe (Figure 6); 8) structure and length of ramenta, and position of their occurrence; 9) the number and size of ‘windows’ on the inside or lower surface of their occurrence (Figure 7); 10) the diameter of bracts. Appendix 1 shows how the measurement was taken in the field.
Figure 6: Morphological measurement of *R. tuan-mudae*. 1) The diameter of aperture (cm); 2) the diameter of whole diaphragm (cm); 3) the diameter of open flower (cm); 4) the number and diameter of perigone lobe (cm); 5) the number of processes; 6) the number and patterns of warts on upper surface of diaphragm; 7) Colour and pattern of perigone lobe.

Figure 7: Inner structure of *R. tuan-mudae*. 8) structure and length of ramenta, and position of their occurrence; 9) the number and size of ‘windows’ on the inside or lower surface of their occurrence.