Assessment on the Phylogenetic Position of *Rafflesia aurantia* with the Endemic Rafflesia Species in the Philippines

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ABSTRACT

Rafflesia aurantia is the most recently discovered Rafflesia that can be found on the Philippines which makes it the only species to not have a phylogenetic position in the phylogenetic tree of the endemic Rafflesia species in the Philippines. The study seeks to provide an analysis on the closest relative of Rafflesia aurantia with the endemic Rafflesia species in the Philippines. Specifically, the study aims to reassess the phylogenetic relationship of Rafflesia aurantia with the endemic rafflesia species in the Philippines in terms of its molecular data and morphoanatomy. The researchers formulated phylogenetic hypotheses on the construction of a new tree that specifies where Rafflesia aurantia is placed on the proposed phylogenetic tree. The researchers used a descriptive quantitative approach to this study and the data gathered were collected through a bibliographic technique and molecular data were gathered from GenBank. The collected FASTA format of nucleotide sequences of the twelve Rafflesia species were placed in the MEGA software with a bootstrap of 100. Significant relationship among species was found even though morphology and molecular analysis are both different factors. This is due to the avoidance of gamete wastage and hybridization which are coping mechanisms of the Rafflesia in order to conserve gene even with the ecological condition. It is found out that using morphological assimilation on determining the phylogenetic position of Rafflesia species are closely correlated to the phylogenetic tree constructed using molecular data of the different species. The data showed that Rafflesia aurantia belongs in the clade between Rafflesia baletei and Rafflesia lobata in terms of its morphology. Since DNA sample of Rafflesia aurantia is not yet available, it is recommended for future researchers to further prove and solidify the proposed position Rafflesia aurantia among the phylogenetic tree of Rafflesia species.

Keywords: *Rafflesia aurantia, endemic rafflesia species, Philippine rafflesia*

INTRODUCTION

Rafflesia (*Rafflesiaceae*) is a holoparasitic plant that is native to the tropical forest of Southeast Asia and parts of Europe. It is identified to have very large flowers and emits a putrid or foul odor that is believed to attract pollinators and agents of seed dispersal (Pelser et al., 2016). The flower's color can be identified or characterised to be reddish-brown or purplish brown, with the sex organs in a central cup. The fruit of a rafflesia is a berry containing very sticky seeds that is thought to be disseminated by fruit-eating mammals and other small animals. Rafflesia species are well known to have an endo-parasitic relationship with their host plants, which are exclusively vines of the genus Tetrastigma or plants in the grape family.

Many Rafflesia species discovered are rare and threatened by habitat destruction and degradation. In 1997, IUCN included many species of Rafflesia to the Red list of Threatened plant species (Barcelona, 2009). Another factor that contributes to the greatly decreasing population of Rafflesia species in the world is also because of environmental factors and the scarcity of Tetrastigma population in which most Rafflesia species depends in order to proliferate. Rafflesia has high island endemism and is caused by poor inter-island dispersal abilities of the plant and sensitivity to habitat and climate change (Bendiksby et al., 2010). The restricted distribution of many Rafflesia species in between different islands or landmasses also indicates that Rafflesia might be dispersal-limited or is very dependent.

A newly discovered species of Rafflesia in the Philippines is Rafflesia aurantia. This species is the ninth species of Rafflesia named from the Philippines, seven of which having been named after 2002. This species most resembled R. tengku-adlinii of Borneo in that both have orange flowers with relatively indistinct markings. It differs from this species, however, in features of the ramenta, disk processes, and anther number. There are currently no species of Rafflesia that occur on both Borneo and the Philippine islands. This suggests that the species have limited dispersal ability and that disjunctions are unlikely (Barcelona, 2009).

Rafflesia aurantia is only known from its type locality in the Quirino Protected Landscape (QPL), Quirino Province on Luzon Island, Philippines. This population occurs in a disturbed lowland mountainforest on the slopes of the Mungiao Mountains near the headwaters of the Cagayan River. The discovery of Rafflesia aurantia in the parts of Sierra Madre Mountains of Luzon significantly highlights the high biodiversity present in this region (Batolome, 2009) The research aims to further develop claims and improve previously published phylogenetic hypotheses and systematic approaches on the diversification of the genus Rafflesia found in the Philippine Archipelago by providing newly constructed phylogenetic tree which is based on morphological aspects and molecular sequence of the different Rafflesia species in the Philippines in order to provide Rafflesia aurantia a phylogenetic position in the newly formulated tree.

Review of Literature

The Genus Rafflesia

Rafflesia, a genus of parasitic plants that lacks leaves, chlorophyll, separate stems and roots, as they are dependent entirely on their host for the nutrients that they need. These can be found in forested lowlands and montane forests of Borneo, Java, Peninsular Malaysia, Philippines, Sumatra, and Thailand (J. F. Barcelona, Pelser, Balete, & Co, 2009). Currently, there are between 30 to 37 species recognized and out of this, 11 are discovered to be present in the Philippines (Pelser, Nickrent, van Ee, & Barcelona, 2019). This kind of plant lives inside their hosts, which are the species of Tetrastigma, a genus of plants in the grape family. Only flowers emerge in the Rafflesia and some are considered to be the largest among all flowering plants, reaching up to 1.5m in diameter (J. Barcelona, Co, Balete, Bartolome, & Balete, 2009).

Conservation Problems and Approaches

One of the discussions of researchers involve the survivability of Rafflesia species. Factors such as failure in pollination processes, the failure in growth of buds, and the infestation of insects contributes greatly to the situation of this plant. Also, its natural absence of chlorophyll makes it too dependent on its host, and as Tetrastigma is a climber, it needs a tree for support in reaching the light above the canopy of the forest, thus, a disturbance in any of these will also affect the survival rate of the Rafflesia.

Most of the conservation problems are natural, and occurences such as typhoons can cause habitat destruction. Destroyed canopies enables the change in temperature, and surface run-offs kill the buds that the plant produces for reproduction. Moreover, species such as the pollinators of Rafflesia can also be affected, further endangering the plant's population.

Approaches are being done in order to prevent such issues and the first step in doing so is to understand the biology of the area or the forest. It is obvious that the only time Rafflesia can thrive is during dry months, the time where typhoons are less likely to occur. Even so, there will still be other natural events, and this would be hard to predict as it can happen at any time. Therefore, the regular monitoring of these species is the best way and most importantly, the raising of awareness of the local community and the visitors, in the way of personal interaction or through researches, of how important Rafflesia is, as it is also an indicator of the ecological state of the mountain or wherever it lives (Yahya et al., 2010).

Evolution of Parasitism

Rafflesia or also known as corpse flower and its host, which is the Tetrastigma, have a very intimate relationship. At first, Rafflesia burrows into the Tetrastigma's roots and stems and burrowing deep into its xylem and phloem. They are very dependent on their host that the Rafflesia have even lost its evolutionary trait to make chlorophyll pigments, a requirement for photosynthesis, and thus debunk the nature of being a true plant by being unable to produce food from sunlight. These parasites feed off their host vines, growing and proliferating until flowers start to bloom into large, rubbery flowers that stink like rotting flesh (Wilcox, 2012).

After generations of intimate contact between parasite and host, Rafflesia species has ended up with more than the usual parasitic traits. As a new study published today in BMC Genomics reveals, the parasite expresses dozens of new genes that it has co-evolved from its host plant. The passage of genes from distant lineages is known as horizontal gene transfer. Though common in bacteria it is much rarer in plants and animals and this can only be caused by millions of years of Co- evolution (Wilcox, 2012).

Objectives of the Study

Generally, the study seeks to provide an analysis on the closest relative of Rafflesia aurantia with the endemic Rafflesia species in the Philippines. Specifically, the study aims to reassess the phylogenetic relationship of Rafflesia aurantia with the endemic Rafflesia Species in the Philippines in terms of its molecular data and morphoanatomy.

METHODOLOGY

The researchers used a descriptive quantitative approach to this study. The data gathered were collected through a bibliographic technique. Data from published data sets were provided through online by Pieter B. Pelser, a lecturer in Plant Systematics, curator of the herbarium at the University of Canterbury in Christchurch, New Zealand and one of the authors of the many literatures used. As for another reference, Gen Bank was used as the primary source of Nucleotide sequences which is in the format of FASTA and is in terms of the 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, 26S ribosomal RNA gene, internal transcribed spacer two of the Rafflesia species. For the construction of the Phylogenetic trees, the collected FASTA format of nucleotide sequences of the twelve Rafflesia species were placed in the MEGA software with a bootstrap of 100. In addition, a geological distribution map of the Rafflesia's presence in the Philippines is created base on literature review of published data sets.

RESULTS AND DISCUSSIONS

The Phylogenetic tree shows that the nucleotide sequences of the Rafflesia species in the Philippines are closely related to each other. Rafflesia mira was the one who speciated first in terms of the common ancestral nucleotide sequence (Pelser et al., 2019). In the same study of Pelser, the biographical relationship of the Rafflesia is considered to be as the main factor of the morphological differences within it. Under the assumption in terms of the divergence time estimation that the colonization of Rafflesia in the Philippines in the Cenozoic development of the Southeast Asia, the Rafflesia population were scattered through different parts of the country namely: Luzon, Visayas, and Mindanao. This is according to the study of Pelser which further stated that the apomorphy of Rafflesia in terms of flower sized do varies yet, its DNA sequence is still closely related. An example of which is that Rafflesia schadenbergiana is the species with the largest flower diameter (J. Barcelona et al., 2009) shows the most closely related in terms of the DNA sequence in the Rafflesia verrucosa which is the species with the smallest flowers (Figure 1).

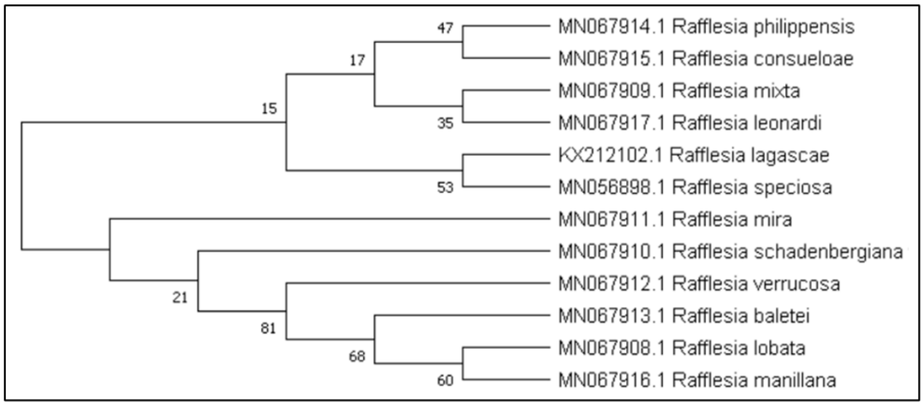

Figure 1. Maximum Likelihood Phylogenic Analysis of Rafflesia species in the Philippines; Bootstrap = 100, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, 26S ribosomal RNA gene, internal transcribed spacer 2 using the MEGA Software

Sympatric Rafflesia species' flower sizes do not go far from each other. This is due to the environmental factor which is the habitat itself and the nutrition that it needs for its own survival (Maher, Richter, & Markewitz, 2007). Still, the molecular relationship of the species remains the same as seen in (Figure 1). Natural selection for character displacement were adapted by the Rafflesia in order to avoid gamete wastage and hybridization. In result to this, the gene sequence of the Rafflesia species is preserved and passed through the next generations (Pelser et al., 2019). In the same case (Figure 1), Figure 2 shows the same genetic relationship of both Rafflesia schadenbergiana and Rafflesia verrucosa which are known to have the biggest and smallest flower sizes (J. F. Barcelona et al., 2009).

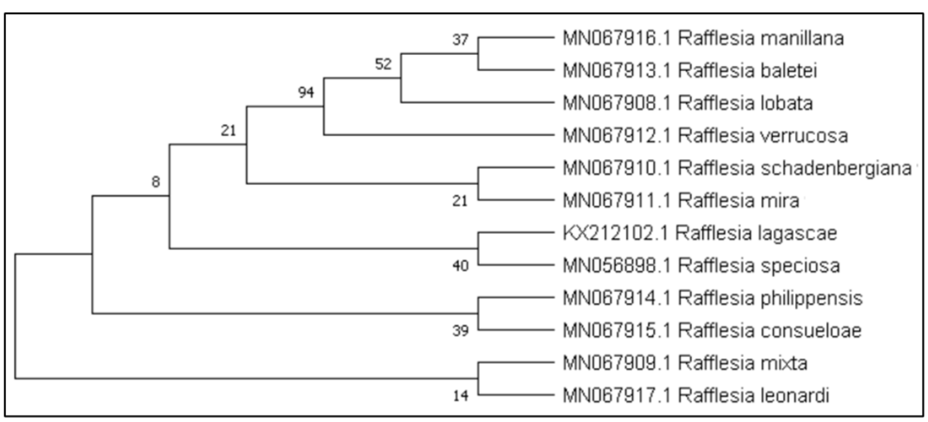

Figure 2. Neighbor Joining Clade of Rafflesia species in the Philippines; Bootstrap = 100, 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, 26S ribosomal RNA gene, internal transcribed spacer 2 using the MEGA Software

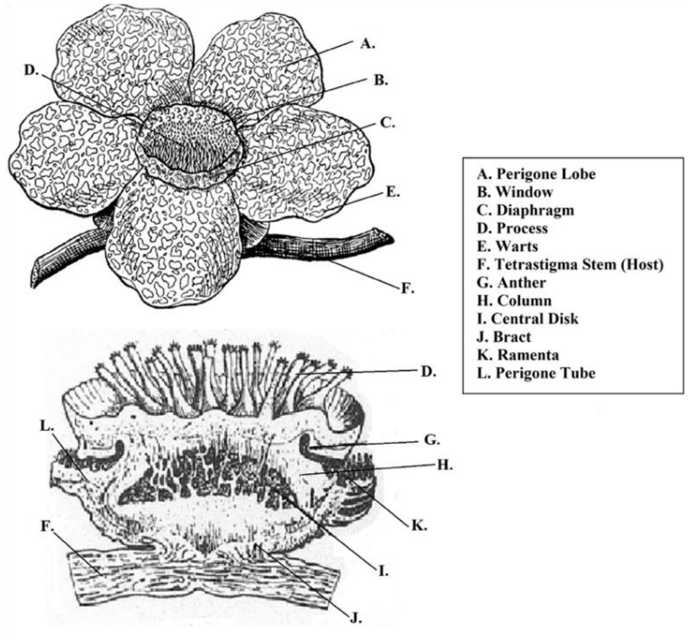

Figure 3. Main Parts of the Rafflesia flower; Courtesy of: Biology 12: Environmental Science. Pp. 12-16. Rex Bookstore

Table 1. Rafflesia in Luzon

	R. consueloae	*R. aurantia*	*R. baletei*	*R. manillana*	*R. lagascae*	*R. philippensis*	*R. leonardi*
FLOWER							
Flower diameter (cm)	6.6 – 12.7 cm	20 cm	9 – 22 cm	11 – 24 cm	14 – 24 cm	17. 5 – 27 cm	25.5 – 50 cm
Perigone color	Reddish orange	Orange	Orange or reddish orange	Reddish orange	Reddish to reddish brown	Reddish orange	Reddish orange
Perigone wart shape	Sharply-edged, areola-forming	Sharply-edged, areola-forming	Round or elliptic	Round or elliptic	Round and elliptic	Elliptic	Round or elliptic
DIAPHRAGM							
Diaphragm color	Darker, rarely same as perigone color	Same as perigone color	Darker	White	White	White	Concolorous
Diaphragm - aperture diameter (cm)	2.04 – 3.30 cm	2.8 – 3.3 cm	2.3 – 2.5 cm	1.33 – 1.8 cm	1.29 – 1.33 cm	2 – 2.2 cm	1.2 – 1.5 cm
DISK							
Disk processes number	10 - 18	Not counted	19 - 26	8 - 30	11-24	Up to 25	Up to 16
Disk processes arrangement	Centrally disposed	Centrally disposed	Regular concentric rings	Irregular concentric rings	No info	Irregular, not in rings	Irregular, concentric rings
Disk process length (cm)	3 cm	1 cm	1 cm	0.55 cm	0.5 cm	1.5 cm	1.2 cm

RAMENTA							
Ramenta color	Slightly darker	Slightly darker	Darker	White	White	Darker	Darker
Ramenta length (mm)	0.5 – 3 mm	7 – 10 mm	Up to 2	0.5 – 1 mm	Up to 2	Up to 5	Up to 2
Ramenta distribution	Thinly scattered	Thinly scattered	Uniform	Thinly scattered	Thinly scattered	Thinly scattered	Abundant
OTHERS							
Windows	absent	absent	absent	Present	Present	Present in large flowers	Absent
Anther Number	12 - 14	12-14	11-14	10 – 18	14 – 18	14 - 16	20 -22

Table 2. Rafflesia in Visayas

	R. lobata	*R. manillana*	*R. speciosa*
FLOWER			
Flower diameter (cm)	11 – 21 cm	11 – 24 cm	45 – 56 cm
Perigone color	Reddish orange	Reddish orange	Reddish orange
Perigone wart shape	Round	Round or elliptic	Elongated
DIAPHRAGM			
Diaphragm color	Concolorous	White	White
Diaphragm aperture diameter (cm)	Up to 1	1.33 – 1.8 cm	1.8 – 2 cm
Diaphragm aperture shape	Round	Round	Round
DISK			
Disk processes number	7 – 14	8 - 30	17 – 31
Disk processes arrangement	Irregular, concentric rings	Irregular concentric rings	Regular concentric rings
Disk process length (cm)	0.5 cm	0.55 cm	1.1
Disk types	Monomorphic	Monomorphic	Tightly packed
RAMENTA			
Ramenta color	White	White	Concolorous
Ramenta length (mm)	1 – 2 mm	0.5 – 1 mm	7 mm
Ramenta distribution	Thinly scattered	Thinly scattered	Uniform
OTHERS			
Windows	present	Present	Absent
Anther Number	10 - 11	10 – 18	20 – 21

Table 3. Rafflesia in Mindanao

	R. verrucosa	*R. mixta*	*R. mira*	*R. schadenbergiana*
FLOWER				
Flower diameter (cm)	14.5 - 16	40-55	45 - 60	52 - 80
Perigone color	Reddish-orange/ cinnamon	Reddish brown	Reddish orange to red	Reddish - orange to maroon
Perigone wart shape	Prominently raised, irregular in shape, usually roundish	Powdery white becoming concolorous with age	Mostly round or eliptic	Elongated to reticulate
DIAPHRAGM				
Diaphragm color	concolorous	concolorous	Reddish orange to red	white
Diaphragm aperture diameter (cm)	1.75 - 2.25	1.8 - 2.3	2	1.8 - 2.2
Diaphragm aperture shape	Round, sometimes irregular	round	round	Round
DISK				

Disk processes number	Not counted	22-29	38-40	30-63
Disk processes arrangement	Irregular, inter-connected system	Regularly disposed	Irregular concentric	Regular concentric
Disk process length (cm)	1.1	1	3.5	3
Disk types	tightly packed, laminar plates with erose margins	dimorphic; conical in center zone; radially flattened at base and more clearly so towards the apex in outer zone	polymorphic in 4 zones: conical central ones, followed by blades perpendicular to each other, the outermost ones reduced	monomorphic, conical, often branched
RAMENTA				
Ramenta color	white-tipped in newly opened flowers, otherwise concolorous	concolorous	darker	darker
Ramenta length (mm)	7	2	5	4-10
Ramenta distribution	uniform	Uniformly dense	sparse at base, abundant halfway, less abundant towards the aperture	sparse at base, abundant halfway, less abundant towards aperture
OTHERS				
Windows	absent	absent	absent	absent
Anther Number	20 - 21	9-20	10-22	26-40

Figure 4 shows the phylogenetic tree of the 13 Rafflesia species in the Philippines based on their morphology and anatomy. The main basis in classifying the species is with their increase in flower size, as according to (Nikolov & Davis, 2017), Rafflesia is related to the family Euphorbiaceae, wherein flowers are small, therefore, the giant flowers present nowadays are believed to be a result of evolution after millions of years. In the same study, a phylogeny of the family Rafflesiaceae was constructed, and they found out that the genera Sapria and Rhizanthes, other groups of parasitic plant, were first to emerge. Rafflesia, being the most advanced in the family, is observed to be exhibiting a maximum size of 80 cm. in floral diameter of one species, while in Sapria and Rhizanthes, flowers vary from 14 – 43 cm only. The quantity of the anthers of the Rafflesia are directly proportional to the increase in flower size except in *R. verrucosa*. Windows are absent for all of the species except in *R. manillana, R. lagascae*, and *R. philippensis* if it is large in size. Unique characteristics are shown for the species *R. consueloae, R. baletei, R. aurantia, R. lobata, R. manillana, R. lagascae,* and *R. philippensis* as indicated on the phylogenetic tree. Moreover, the *R. manillana* and *R. lagascae* were put together in one branch as they were previously considered as synonyms due to their mostly similar morphological characteristics (Pelser, Nickrent, Callado, & Barcelona, 2013). While for the *R. speciosa* and R. *mira*, they have the same initial size and closely related final size when it comes to floral diameter.

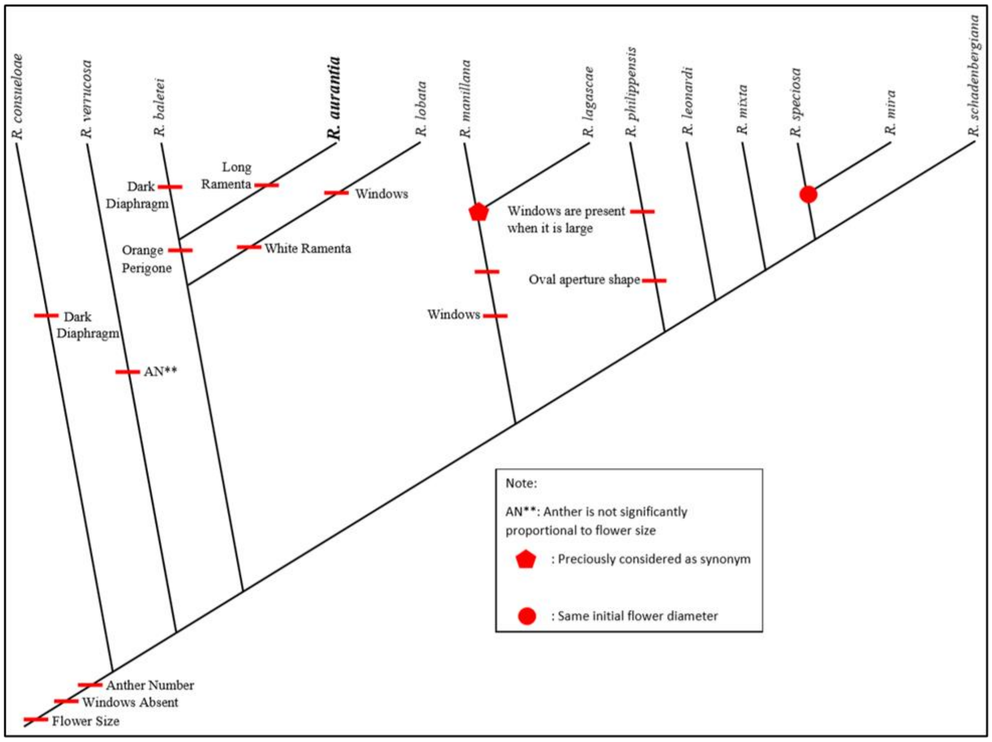

Figure 4. Phylogenetic Position of Rafflesia aurantia based on morphology

An inverse relationship between its floral size and latitudinal distribution has also been observed. According to (Bascos, Rodriguez, Duya, Fernando, & Ong, 2019), there is an increase in the flower diameter as the latitude goes down (Luzon to Mindanao), but in exception to the species R. verrucosa and R. leonardi which might be due to environmental or speciation factors. Sympatric Rafflesia species often share the same size despite the different species that they belong to, while Allopatric Rafflesia species differ in sizes even though they are of the same species. As a result to this morphological phylogenetic tree, a hypothetical molecular phylogenetic position of Rafflesia aurantia is shown in Figures 5 and 6.

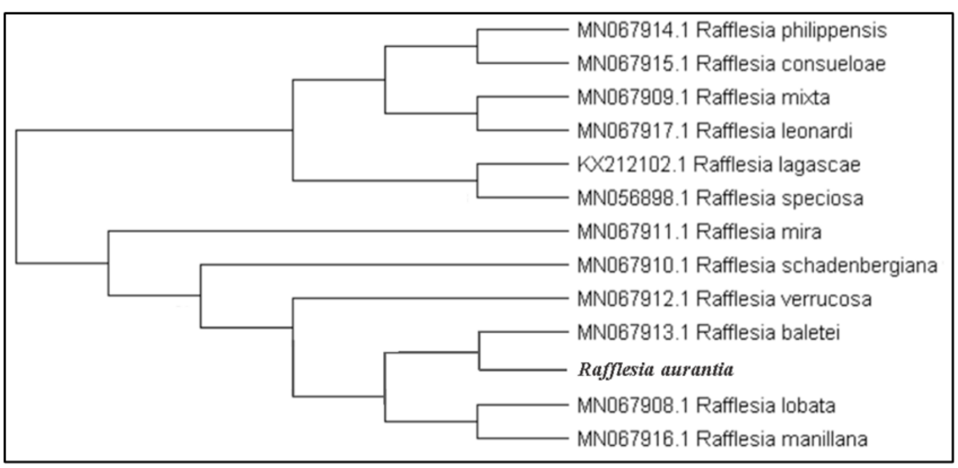

Figure 5. Hypothetical Molecular Phylogenetic Position of Rafflesia aurantia based on morphology; MEGA Software (Maximum Likelihood)

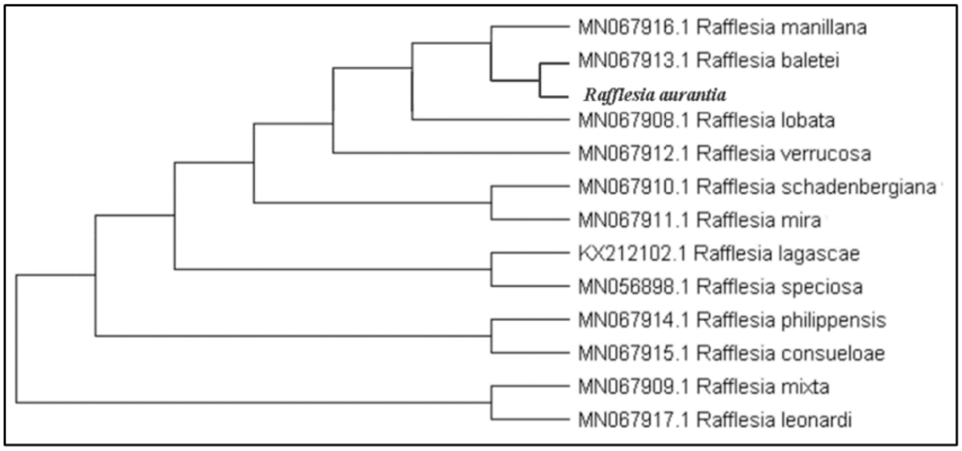

Figure 6. Hypothetical Molecular Phylogenetic Position of Rafflesia aurantia based on morphology; MEGA Software (Neighbor Joining)

CONCLUSIONS

The study has shown that *Rafflesia aurantia,* although still lacking for its nucleotide sequence in terms of its 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, 26S ribosomal RNA gene, and internal transcribed spacer 2, it is hypothetically genetically related to the *Rafflesia baletei* and *Rafflesia lobata* with the basis on its morphology. It is found out that the Rafflesia species can transfer closely related gene through generations even with the change of its morphology. This explains the reason behind why *Rafflesia aurantia* still lacks its DNA sequence.

Moreover, an inversely proportional relationship between floral size and latitudinal distribution has been shown in the study. From Luzon to Mindanao, the floral diameter of the Rafflesia species increases. In consideration of the shared unique morphological characteristics as well, the phylogenetic relationship of *Rafflesia aurantia* with the rest of the endemic Rafflesia species in the Philippines has been reassessed, which led to it being hypothetically positioned in the clade between *Rafflesia baletei* and *Rafflesia lobata*.

For future researchers, they could extend the research through finding the DNA sequence of the *Rafflesia aurantia* by gathering physical sample and determining the DNA sequence in terms of its 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, 26S ribosomal RNA gene, and internal transcribed spacer 2 (Gen Bank). This will further prove the phylogenetic position of the *Rafflesia aurantia* with the molecular phylogenetic basis since the data for morphology is available.

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