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Review

Dissecting the Biology of *Rafflesia* Species: Current Progress and Future Directions Made Possible with High-Throughput Sequencing Data

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The angiosperm *Rafflesia* exhibits a unique biology, including a growth strategy that involves endophytic parasitism of a specific host, with only the gigantic flower externally visible. The *Rafflesia* possesses many unique evolutionary, developmental and morphological features that are rooted in yet-to-be-explained physiological processes. Although studies on the molecular biology of *Rafflesia* are limited by sampling difficulties due to its rarity in the wild and the short life span of its flower, current advances in high-throughput sequencing technology have allowed for the genome- and transcriptome-level dissection of the molecular mechanisms behind the unique characteristics of this parasitic plant. In this review, we summarize major findings on the cryptic biology of *Rafflesia* and provide insights into future research directions. The wealth of data obtained can improve our understanding of *Rafflesia* species and contribute toward the conservation strategy of this endangered plant.

Keywords: Genome • Host–parasite interaction • Parasitic plant • Phylogeny • *Rafflesia* • Taxonomy • Transcriptome

Introduction

Despite having been discovered more than two centuries ago during an expedition by Sir Stamford Raffles in 1818 (Brown 1822), the *Rafflesia* remains an enigma to science. A deep mechanistic understanding of the molecular biology for its flower gigantism and the evolution of its parasitism still eludes us. *Rafflesia* belongs to the Rafflesiaceae family of holoparasitic plants that depend entirely on their host plants for fixed carbon and other nutrition requirements (Meijer 1993, Nais 2001). The Rafflesiaceae family is divided into three genera that include *Rafflesia*, *Rhizanthes* and *Sapria*. *Rafflesia* is well-known for producing the largest flowers in the world, with *Rafflesia arnoldii* being on record for having a flower of >100 cm in diameter (Meijer 1984, Nais 2001, Barkman et al. 2008). Members of this genus are only found in the pristine rainforests of Southeast Asia within the borders of Malaysia, Java, Sumatera, Anabas Island, Kalimantan (Indonesia), Brunei, the Philippines and Thailand (Wong et al. 2009, Balete et al. 2010). A feature of *Rafflesia* that draws the most attention is perhaps its enormous and vivid flowers that are devoid of any visible leaves, stems and roots (**Fig. 1A**). The flower's large reddish petal–like perigone lobes are sprinkled with white blotches; this coloration has been considered as an attractive visual indicator that plays an important role in its survival and reproduction, as expected of fly-pollinated flowers (Davis et al. 2008). In addition to the visual cues, the presence of several unicellular types of trichomes and multicellular ramenta, a slender structure with capitate and branched tips, on the inner surface of the flower's cavity may also play a role in directing pollinators to the anther chamber of the male flower or the stigmatic area of the female flower (Beaman et al. 1988, Nais 2001, Nikolov et al. 2014a). *Rafflesia* has an identical floral architecture to *Sapria*, but their perianths differ, where *Rafflesia* has one whorl of five and rarely up to 10 perianth lobes (petal-like structures) and *Sapria* has two whorls each with five similar lobes.

The other lesser-known genera, *Rhizanthes* and *Sapria*, also exhibit similar peculiar morphological characteristics despite being much more diminutive in size (14 cm and 20 cm average flower diameter, respectively). In terms of physical appearance, *Sapria* is most similar to *Rafflesia* due to its vermilion-colored flowers being covered with sulfur-yellow spots that consist of 10-lobed perigones that are separated as an outer and inner series (Trần et al. 2018). Conversely, the most significant difference between *Rafflesia* and *Sapria* is the absence of a fully developed diaphragm, an organ where the floor and walls of this chamber are formed by a perianth tube and the roof. Unlike the other two genera, the *Rhizanthes* flower developed calli at the place where the diaphragm would be found (Meijer and Veldkamp 1988). Based on the geographic distributions, *Sapria*

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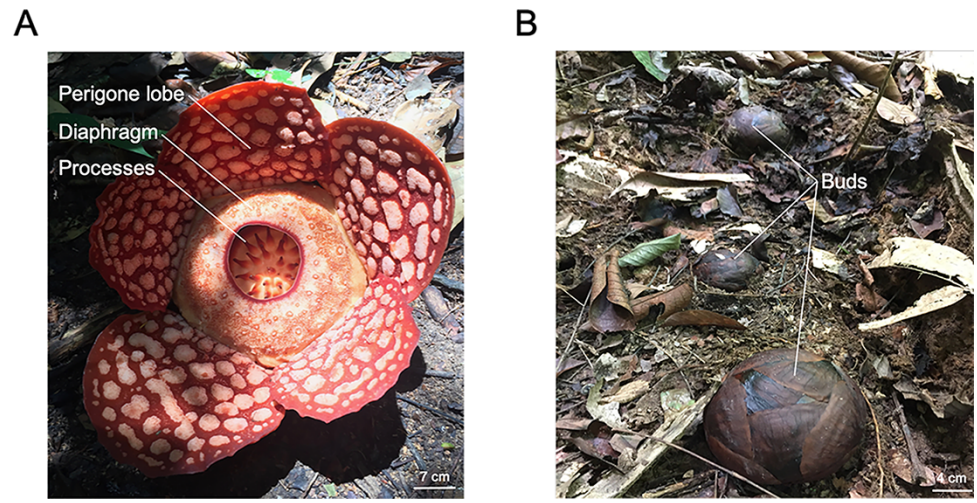

Fig. 1 *Rafflesia cantleyi* sighted at Royal Belum Reserves Forest, Perak, Malaysia. (A) A blooming flower. (B) Developing buds; the host vine is obscured by the soil.

Table 1 Sequencing-related studies on *Rafflesia*

Research focus	Types of sequence data	Technology	Reference
Phylogeny	Mitochondrial gene (*matR*)	Sanger sequencing of PCR fragments	Barkman et al. (2004)
Evolutionary biology and genomic chimerism	Mitochondrial genes (*atp1*, *cox1* and *matR*)	Sanger sequencing of PCR fragments	Barkman et al. (2007)
HGT	Floral transcriptome	Illumina sequencing of cDNA transcripts	Xi et al. (2012)
Mitochondrial gene transfer	Mitochondrial genome	Illumina sequencing of genomic DNA	Xi et al. (2013)
Possible loss of chloroplast genome	Whole genome	Illumina sequencing of genomic DNA	Molina et al. (2014)
Flower development	Floral transcriptome	Illumina sequencing of cDNA transcripts	Lee et al. (2016)
Host specificity	Nuclear and plastid DNA	Sanger sequencing of PCR fragments	Pelser et al. (2016)
Flower development	Transcriptome of floral bud stages	Illumina sequencing of cDNA transcripts	Amini et al. (2017)
Deletion or loss of plastid genes	Whole-genome and floral transcriptome	Roche 454 sequencing of genomic DNA and Illumina sequencing of cDNA transcripts	Ng et al. (2018)
Flower development	Transcriptome of floral bud stages	Illumina sequencing of cDNA transcripts	Amini et al. (2019)
Flower senescence	Transcriptome of flower stages	Illumina sequencing of cDNA transcripts	Mohd-Elias et al. (2021)
Species differentiation	Mitochondrial genome	Illumina sequencing of genomic DNA	Chin et al. (2022)

is found to be more prevalent in the upper parts of Southeast Asia and spread up to the Himalayan region in northeast India, while *Rhizanthes* is more commonly found in the lower parts of Southeast Asia, including the Malay Peninsula, Bornean, Javan and Sumatran regions (Bänziger and Hansen 2000).

In recent years, there has been an increase in the number of molecular-level studies to investigate *Rafflesia* biology. These include several that involved DNA sequencing of *Rafflesia* genes (**Table 1**). However, today's complex research questions require a volume and depth of information beyond the capacity of traditional DNA sequencing technologies. High-throughput sequencing has filled that gap to become an indispensable research tool to address the many questions on the biology of *Rafflesia*. These functional genomic and transcriptomic studies on *Rafflesia* can provide key evidence for understanding the evolutionary novelty and heterotrophic ability of *Rafflesia* as a parasitic plant. In this paper, we review the literature published on various aspects of *Rafflesia* biology, including its taxonomy and phylogeny, development, reproduction and host–parasite interaction, and present the important research gaps that need to be explored using high-throughput sequencing data.

Taxonomy and Phylogeny of *Rafflesia* Species

The taxonomy and phylogenetic resemblance of *Rafflesia* are indistinct due to its odd morphology and evolution as endophytic holoparasites. Initially, *Rafflesia* was grouped with other parasitic plants such as *Apodanthus*, *Pilostyles*, *Cytinus*, *Bdallophytum* and *Mitrastema* in various taxonomic treatments (Meijer 1997). Subsequently, comparisons of the mitochondrial gene *matR* of 95 angiosperm and gymnosperm species placed Rafflesiaceae as a member of Malpighiales with sister families such as Passifloraceae, Salicaceae and Violaceae (Barkman et al. 2004, 2007, Wurdack and Davis 2009). Further analysis using five mitochondrial and one chloroplastic genes from species of all Malpighiales families was able to assertively place Rafflesiaceae within the Malpighiales as nested in Euphorbiaceae

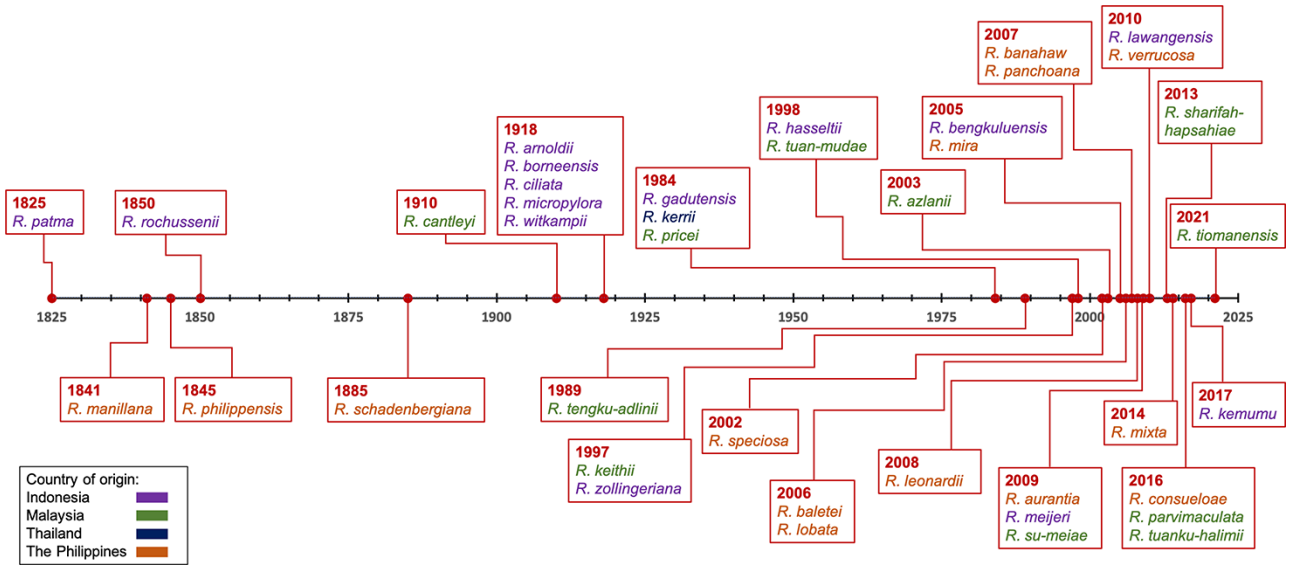

Fig. 2 Timeline of the discovery of *Rafflesia* species. Information was compiled using reports from the following references: *R. arnoldii, R. cantleyi, R. gadutensis, R. hasseltii, R. keithii, R. manillana, R. micropylora, R. patma, R. pricei, R. rochussenii, R. schadenbergiana, R. tengku-adlinii, R. tuan-mudae, R. zollingeriana, R. witkampii, R. ciliata, R. borneensis* and *R. philippensis* (Meijer 1997); *R. su-meiae* (Wong et al. 2009); *R. azlanii* (Latiff and Wong 2003); *R. sharifah-hapsahiae* (Adam et al. 2013); *R. parvimaculata* (Sofiyanti et al. 2016); *R. tuanku-halimii* (Adam et al. 2016); *R. tiomanensis* (Siti-Munirah et al. 2021); *R. bengkuluensis* (Susatya et al. 2005); *R. meijeri* (Wiriadinata and Sari 2010); *R. lawangensis* (Mat-Salleh et al. 2010); *R. kemumu* (Susatya et al. 2017b); *R. speciosa* (Barcelona and Fernando 2002); *R. mira* (Fernando and Ong 2005); *R. baletei* (Barcelona et al. 2006); *R. lobata* (Barcelona et al. 2006); *R. panchoana* (Madulid et al. 2007); *R. banahaw* (Barcelona et al. 2007); *R. leonardii* (Barcelona et al. 2008); *R. aurantia* (Barcelona et al. 2009); *R. verrucosa* (Balete et al. 2010); *R. mixta* (Barcelona et al. 2014); *R. consueloae* (Galindon et al. 2016) and *R. kerrii* (Meijer 1984).

(Davis et al. 2007). These studies proposed a prompt evolution leading to highly specialized and uncommon floral morphology. In addition, phylogenetic studies also revealed that the nature of floral gigantism in *Rafflesia* is relatively recent and rapid with the floral diameter increasing an average of 20 cm/million years (Davis et al. 2007, Barkman et al. 2008, Davis 2008). Bendiksby et al. (2010) also reported that the evolutionary history, specifically the floral morphology of *Rafflesia*, was influenced by the rainforest-favorable conditions of the Mid-Miocene to Pliocene epochs.

A conclusive number of *Rafflesia* species are yet to be determined due to its rarity in remote habitats and total dependence on its host plant. Some species have not been documented for many years and may be facing extinction if not already extinct. Several species described were not acknowledged due to being synonyms of previously described species and scarcity of supporting data. The characterization of these species was also based on immature or incomplete buds due to the lack of well-preserved specimens (Meijer 1997). Up to a total number of 40 species have since been recorded from Indonesia, the Philippines, Malaysia and Thailand (**Fig. 2**).

The taxonomic legitimacy of several species has been raised mainly due to the inefficient and complex morphological characterization of the species. For *Rafflesia*, taxonomic speciation is based on the morphology of the flowers, with most emphasis being on the outer appearances such as the ramenta's structure, including their location and density in the perigone tube, and on the diaphragm. However, insufficient description of the organ structure has led to difficulties in such purely morphological descriptions of the species (Susatya et al. 2017a). Thus, a better characterization of the species, including their current distribution and taxonomy, particularly of the little-known species, is desperately needed.

Molecular approaches based on mitochondrial genome sequences can provide an effective species classification method. Several mitochondrial genes, namely, *cob, rp110, mttB* and *ccmB*, have been shown to display different orientations among *Rafflesia* species, while the *nad1* gene sequence showed differences among the *Rafflesia* species studied (Chin et al. 2022). This indicates that mitochondrial gene sequences could be useful in the development of molecular markers for *Rafflesia* species classification. Mitochondrial genome sequence data generated using high-throughput sequencing could be expanded to scrutinize the origins of different species of *Rafflesia* from different distribution locations, which can lead to a deeper understanding of the relationship between mutations in the sequence and the geographical diversity of the species.

Development of *Rafflesia*

Rafflesia goes through several developmental phases, including growth in the host plant, the development of the flower

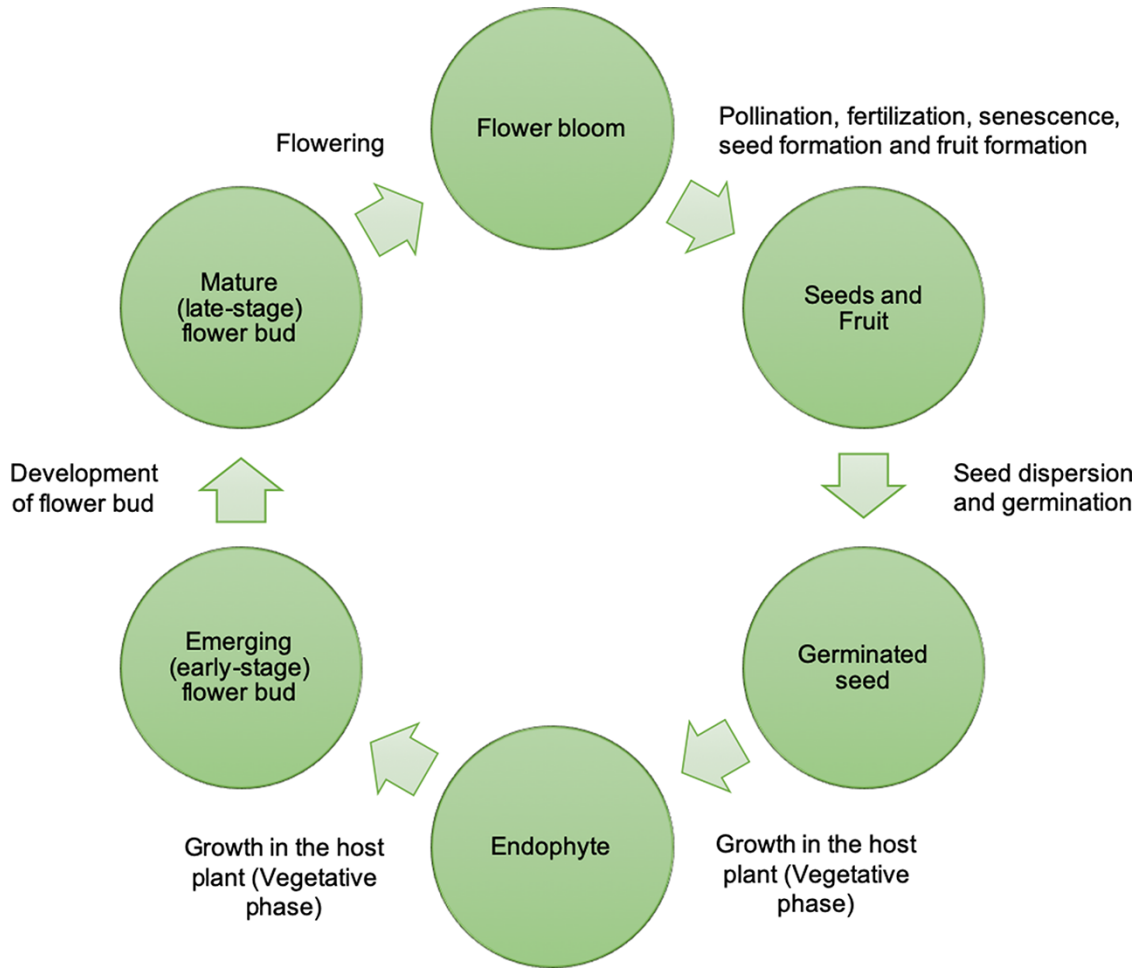

Fig. 3 Life cycle of *Rafflesia*.

bud, flowering and flower senescence (**Fig. 3**) (Nais 2001). The estimated length of the life cycle of *Rafflesia* ranges from 3 to 5 years, depending on the size of the flower (Meijer 1997, Nais 2001). Detailed studies have reported that the lifespan from the current generation seed to the next generation seed for *R. patma* is 3–4 years (Hidayati et al. 2000), while for *R. arnoldii*, it is 3–5 years (Meijer 1997, Susatya 2020). *Rafflesia rochussenii* takes about 2 years to develop from visible bud to ripe fruit (Zuhud et al. 1994).

Growth in the host plant

This unique plant maintains a long endophytic state that can abruptly transition to the flowering stage without forming an elaborate vegetative body (Nikolov et al. 2014b). The vegetative phase in the host plant exists as a filament that resembles a substance called endophytes (Kuijt 1969, Nikolov et al. 2014b). A recent study by Wicaksono et al. (2021) reported that the *Rafflesia* endophyte forms a clonal network of vegetative meristematic cells, separated by the dividing host tissue where each meristematic cell cluster ultimately developing into the primordial floral bud or protocorm. The exact amount of time during which *Rafflesia* is in the vegetative phase remains unknown. However, the continuous flowering from the same host plant suggests that an extensive amount of time is spent in the vegetative phase (Nikolov and Davis 2017).

Development of the flower bud

Rafflesia buds are commonly found attached to the vines on the ground (**Fig. 1B**). The bud development phase can be divided into two stages. The first stage is when the bud emerges from the host plant, while the second stage is the mature bud (Nais and Wilcock 1998). The bud growth has been recorded to take about 12–16 months followed by the flowering phase (Nais and Wilcock 1998). Mohamed and Noor (2016) reported *R. azlanii* as a fast-growing plant where the species only takes about 40–50 d from being a newly emerged bud to a full-bloomed flower. For the buds of *R. patma*, several differences have been observed between the early and late stages of development (Mursidawati and Wicaksono 2020). The early-stage bud consists of three types of cells—densely packed meristematic cells in the distal region, non-elongated parenchyma cells in the middle region and elongated parenchyma cells in the proximal region.

The late-stage bud initially develops the primordial central disc, and this is followed by the primordial bract and perigone lobes.

A recent study based on the comparison of transcriptomes from several flower bud stages revealed that members of several transcription factor families such as WRKY, NAC, bHLH and MYB were differentially expressed during flower bud development in *R. cantleyi* (Amini et al. 2019). Genes involved in different phytohormone signal transduction events such as auxin, gibberellin and cytokinin biosynthesis were also found to be differentially regulated during flower bud development. Moreover, the characterization of several gene families including Auxin Response Factor, AUX/IAA and 14-3-3 gene families implies their importance in the growth and development of *R. cantleyi* (Elias et al. 2016, Rosli and Wan 2018). These studies have shed light on the essential genes and pathways involved in the related processes. Further molecular studies, including on other developmental stages, will increase our understanding at the molecular level of the development of this plant species.

Flowering

Rafflesia blooms can be found all year round and are not seasonal (Nais and Wilcock 1998). However, it is worth noting that seasonality has been observed in *Rafflesia* species in the northerly regions of their range (the Philippines and Thailand) compared to the documented equatorial species (Nais 2001). The more distinct periods in day length and seasonal differences in the higher latitudes may be responsible for this mild seasonality. Although the physiological processes associated with the observed seasonality of the northerly *Rafflesia* are yet to be determined, other studies have in part managed to shed light on the flowering-associated genes in general.

Ramamoorthy et al. (2013) had successfully isolated and characterized the MADS-box gene, *RcMADS1*, from *R. cantleyi*. It was proposed that the *RcMADS1* gene has an orthologous function associated with AGAMOUS-LIKE 24 that plays a role in flower meristem formation and flower blooming. A transcriptome-level analysis of the *Rafflesia* flower had also led to a better understanding of the genes involved during the *Rafflesia* flowering process (Lee et al. 2016). Among the genes reported to be highly expressed in the flower include those that code for a sufE-like protein that coordinates iron–sulfur cluster biogenesis and functions in NAD biosynthesis; fructose biphosphate aldolase that plays an important role in sugar production, and hormone and stress signaling; and late embryogenesis abundant protein that protects plant cells under stress conditions. Several genes that are involved in stress response, cellular signaling, cell wall formation and transcriptional regulation were also shown to have higher expression levels in the flower compared to the bud (Lee et al. 2016).

Flower senescence

The *Rafflesia* plant enters the senescence phase when the flower starts to wither, and eventually, death occurs when the entire flower decomposes. The RNA-seq data from two stages of the *R. cantleyi* flower revealed the activation of senescence-associated genes, suggesting a model of flower senescence involving the regulation of ethylene biosynthesis genes and transcription factors for the initiation of senescence and subsequently nutrient remobilization and redox activity that lead to floral organ death (Mohd-Elias et al. 2021). Senescence is crucial in *Rafflesia*, more so as the plant is present almost entirely as a single flower; thus, senescence of the single organ correlates directly with plant death.

Reproduction in *Rafflesia*

The reproductive success in *Rafflesia* is generally low. This may be due to their scarcity, low percentage of buds reaching maturity, limited anthesis period, a large sex inequality and infrequent incidence of the concurrent blooming of both male and female flowers (Nais 2001). Several species of *Rafflesia* have been reported to undergo agamospermy where seeds are produced without going through the pollination process (Meijer 1997, Nais 2001). However, there are limited data to support this claim. The currently available knowledge on *Rafflesia* reproduction presents a clear gap that needs to be filled in order to better formulate strategies for the conservation of this genus. We expect that filling these gaps with data from high-throughput sequencing, including transcriptome datasets, will enable the identification of key genetic components involved in the physiological processes of *Rafflesia* reproduction, thus making such datasets an important contribution toward the conservation of the genus.

Pollination

The *Rafflesia* flower attracts pollinators through sticky yellow pollens and secondary attractors such as odors and visuals (Beaman et al. 1988). Previous studies have recorded carrion fly species as one of the pollinators of *Rafflesia* (Justesen 1922, Meijer 1958, Beaman et al. 1988). *Lucilia* and *Chrysomya* were described to be pollinators for *R. pricei* (Beaman et al. 1988), whereas *Sarcophaga, Chrysomya, Lucilia* and *Hypopygiopsis* have been associated with *R. kerrii* (Bänziger 1991, 2004, Hor et al. 2021) and *R. patma* (Hidayati et al. 2000) pollination. Five more genera of flies visiting *R. patma* have also been reported (Kahono et al. 2010). *Rafflesia cantleyi* flowers produce volatiles that are essential in enticing flies of the species *Chrysomya chani* (Wee et al. 2018). The flower biochemically imitates the carrion, and the oligo sulfides in the floral scent play a vital role in attracting female *C. chani* to the flower (Wee et al. 2018). Zain et al. (2020) also suggested that the presence of phytochemical compounds and a combination of massive floral volatile constituents are thought to contribute to scent emission for attracting pollinators. However, the scent alone is unlikely to be sufficient for inducing landing behavior on the flower. This raises questions on whether other sensory modalities such as touch and vision are also registered by the pollinators.

Seed dispersion

The *Rafflesia*'s female flowers produce hundreds of thousands of ovules (Nikolov et al. 2014a) where it was reported that a fruit from *R. keithii* can produce approximately 270,000 seeds (Nais 2001). Many dispersal agents have been recorded to play a role in distributing these seeds to the *Tetrastigma* (Vitaceae) host; these include ground squirrels, wild pigs (Meijer 1958, Bouman and Meijer 1994, Zuhud et al. 1994, Nais 2001), ants, termites, pangolins (Justesen 1922, Kuijt 1969) and elephants (Kuijt 1969). Adult tree shrews and adult plantain squirrels have been observed to feed on the fruit of *R. keithii* (Emmons et al. 1991) and thus may also play a role in seed dispersion. There are currently two hypotheses regarding seed dispersal and host infection where Bänziger (1991) opined that soil fauna serves as the main agents for new host infection, while Justesen (1922) considered that infection takes place on the underground part of the host plant.

Interaction of *Rafflesia* with Its Host Plant

Rafflesia has been reported to parasitize several species of *Tetrastigma*, namely, *T. curtisii* (Ridl.) Suesseng., *T. diepenhorstii* (Miq.) Latiff, *T. hookeri* (Lawson) Planch., *T. glabratum* (Blume) Planch., *T. papillosum* (Blume) Planch. and *T. rafflesiae* (Miq.) Planch. (Niyomdham and Kubat 1987, Meijer 1997, Latiff 2001, Nais 2001, Nasihah et al. 2016). Several studies on the genetic diversity of resistance or tolerance of host plants toward parasitic plants in agricultural systems have been previously conducted (Parker and Riches 1993). Currently available *Tetrastigma* sequence data were able to offer key information that allowed for the determination of potential genetic markers to classify species that are susceptible to infection (Hafizzudin-Fedeli et al. 2022). Sequences associated with chloroplast and mitochondrial genes can be developed as markers to differentiate *T. rafflesiae* from other *Tetrastigma* species. The utilization of DNA barcoding could also be a useful means of studying the ecological evolution, geographical distribution and population of the host species.

The evidence from RNA-seq analysis by Ichihashi et al. (2015) demonstrated the lack of photosynthetic capacity in parasitic plants. Due to their parasitic lifestyle, *Rafflesia* also does not need to maintain any autotrophic capacity, thus negating the requirement for a functional photosynthetic body. Molina et al. (2014) suggested that *Rafflesia* may be the first plant group where the chloroplast genome is absent. Despite its plastid genome reduction or absence, several chloroplastic genes are still present in the nuclear genome and have been reported to be expressed to fulfill essential biological functions not related to photosynthesis (Ng et al. 2018). Although acquisition of such genes from the host is possible, the phylogeny of *Rafflesia* for two selected nucleus-encoded proteins, riboflavin synthase and lysophosphatidic acid acyltransferase that are involved in riboflavin metabolism and phosphatidic acid biosynthesis, respectively, revealed no evidence that the genes encoding those functions were acquired from its host (Ng et al. 2018). Such a phenomenon is not unique to *Rafflesia* as it has also been observed for other plants (Schein et al. 2001, de Koning and Keeling 2004).

Although the nuclear-encoded photosynthesis-associated genes previously discussed are not horizontally transferred from the host, several studies have indicated that horizontal gene transfer (HGT) from the *Tetrastigma* host to the *Rafflesia* genomes can indeed occur, thus implying that *Rafflesia* is an HGT acceptor (Barkman et al. 2004, Xi et al. 2012, 2013, Molina et al. 2014, Krause 2015). A phylogenomic study found that 49 out of the 2,316 transcripts analyzed (2.1%) in *R. cantleyi* were likely acquired from its host plant (Xi et al. 2012). Depending on the species, Xi et al. (2013) conservatively indicated that up to 41% of the mitochondrial gene sequences show evidence of HGT in Rafflesiaceae. In *Rafflesia*, HGT involves an extensive collection of cellular functions, including metabolism, respiration and protein turnover (Xi et al. 2012).

Rafflesia is linked to its host plant through the haustorium, which bridges between the parasite and the host, makes a vascular connection and facilitates the transfer of nutrients and other molecules (Twyford 2018). The mode of host–parasite interactions in several other parasitic plants has also been characterized, and it was reported that plants of the genus *Cuscuta* gained organic and inorganic nutrients including water from the host plant through a haustorium (Funk et al. 2007) in a similar fashion to *Rafflesia*. Several factors influence parasitism such as the ratio of parasite biomass to the host, the number of parasites growing on a single host plant, the time taken for a complete parasite life cycle and the potential mutual evolution between the two species (Nickrent 2002). However, a study involving *Pilostyle ingae* and its host, *Mimosa naguirei*, showed that the parasite's dependence on its host did not affect the yield and quality of fruit and seed germination despite the smaller fruit size (Gomes and Fernandes 1994). Several studies were also focused on the production of effective protocols to prevent the invasion of parasitic plants such as *Striga* and *Orobanche* that have led to a reduction in the agricultural production of corn, wheat and other staple crops in Africa.

Biochemical analysis of 2,6-dimethoxy-p-benzoquinonone (DMBQ), a component in Orobanchaceae parasitic plants identified from the actual host roots (Chang and Lynn 1986), and its analogs suggests that semiquinone intermediates formed during redox cycling between quinone and hydroquinone states could trigger haustorium development (Smith et al. 1996). Interestingly, DMBQ has been shown to induce haustorium formation in members of the Orobanchaceae family such as *Triphysaria* species, *Striga* species, *Agalinis purpurea* and *Phtheirospermum japonicum* (Chang and Lynn 1986, Stranger et al. 1995, Smith et al. 1996, Jamison and Yoder 2001, Matvienko et al. 2001, Ishida et al. 2011). Information derived from the studies of plant species that have a similar parasitic lifestyle can be used to direct high-throughput sequencing data analysis for revealing the molecular mechanisms of processes unique to parasitic plants such as haustorium evolution and modification of interactions between plants. Further transcriptomics

studies may then be able to reveal the gene regulatory networks involved in host recognition among the species and could lead to methods or compounds that can disrupt haustorium formation.

The 'dual RNA-sequencing' approach has been used to simultaneously monitor changes in gene expression of both parasite and host (Westermann et al. 2012). This technique, which has been applied in studies of various parasites and their hosts (Choi et al. 2014, Pittman et al. 2014, Petitot et al. 2016), is a promising high-throughput sequencing approach to uncover the interaction between *Rafflesia* and *Tetrastigma*. The genetic information from the dual RNA data of the *Rafflesia* haustorium and its host tissue can provide valuable insights into the genes and pathways that are essential for maintaining the delicately balanced relationship between the parasitic plant and its host.

The progress of high-throughput sequencing technologies has delivered a novel means to study the wide and complex molecular–level impact of host–parasite interactions beyond the previously available capacity of merely characterizing the expression of a single gene under different conditions. Even though such approaches might not provide proof of function, they can direct subsequent functional studies onto promising targets that were previously unknown (Wayne and McIntyre 2002). Genomic analyses also allow for the interdependent genetic framework of parasite virulence and host resistance to be examined. In this aspect, signals such as expansions or reductions of gene families of putative pathogen effectors and associated host targets can be scrutinized (Schrider et al. 2013). Transcriptomic studies can also be aimed to illustrate the expressed fragment of the genome and how it is adopted within an organism. In addition, these studies will provide gene sequence information and expression profiles, and discover novel genes and splice variants (Mortazavi et al. 2008).

The roles of certain genes in the host's immune response can also be explored using high-throughput sequencing data. Behrens et al. (2014) stated that the host immune response differs substantively from the infection route of the pathogen, while Riddell et al. (2014) reported that the expression and alternative splicing of host immune genes are related to parasite genotypes. A study by Foth et al. (2014), which focused on the genome and transcriptome analysis of the vertebrate parasite from the whipworm genus *Trichuris*, revealed the important roles of the chymotrypsin A–like serine protease gene family in parasitism. A recent study on the *Sapria himalayana* genome by Cai et al. (2021) discovered that about 13.2% of gene loss consists of genes that are related to photosynthesis, defense and stress response, and may represent a genetic response for the transition from an autotrophic to a heterotrophic way of life. Driven by hypotheses that functions such as these could be orthologous, such existing datasets can in turn be used to mine *Rafflesia* datasets. Plants have complex defense mechanisms that can be condensed when pathogens or parasitic plants interfere with one of the various processes required for host defense. The volume of data produced by these technologies is a challenge for researchers to extract insightful functional details, yet their potential for enlightening the field of host–parasite interactions in *Rafflesia* species is immense.

Challenges and Future Directions

Despite the rapid progress made within the past decade in understanding the biology of *Rafflesia*, many questions remain unanswered, and many aspects are still unexplored. DNA sequencing was introduced to study genetic variation between *Rafflesia* species in the early 2000s. High-throughput technologies and bioinformatics analyses further revealed the molecular aspects of taxonomy and phylogeny, developmental biology, HGT, flower development and senescence in *Rafflesia*.

Cultivation of *Rafflesia* remains a major challenge despite various attempts to artificially propagate the plant. For example, although many efforts have been made, *Rafflesia* tissue culture is yet to be successfully established (Wicaksono and da Silva 2015), while attempts to artificially sow *Rafflesia* seeds in the *Tetrastigma* stem failed to produce any germination (Mursidawati et al. 2015). Thus, the main challenge in studying *Rafflesia* will continue to be the difficulty of sampling the species in their wild habitat. *Rafflesia* flowers at the different stages are extremely difficult to find especially due to the short blooming period and the lack of information regarding the occurrences of such events in the field. In addition, field samplings carried out suggest that *Rafflesia* buds have a high mortality rate due to the lack of nutrients from the host plant and attacks from herbivores, pathogens and human activities. These observations suggest that the formation of *Rafflesia* flowers should occur at the right time as well as in a conducive environment to ensure the success of the species' life cycle. *Rafflesia* is considered a highly endangered plant species due to its limited distribution, requirement for a specific host plant species and large sex imbalance, as well as the increased threat from rapid habitat loss due to tropical rainforest degradation.

To our knowledge, there are currently no available datasets generated using long-read DNA sequencing technologies such as single-molecule real-time (SMRT) sequencing for *Rafflesia*. The data provided by these advanced sequencing technologies can produce better genome sequence assemblies for comparative analysis of the various species of *Rafflesia* and other members of Rafflesiaceae. Furthermore, the SMRT sequencing technology offers additional information on DNA methylation. This in turn provides an additional layer of data that can reveal insights into regulatory mechanisms that utilize non-protein-coding RNA and methylation state expression control in *Rafflesia*. More extensive experiments can be conducted in the coming years to focus on the analysis of genomes and transcriptomes of different *Rafflesia* species over several generations. This means that unique mutations and modifications in gene expression can be studied in greater detail, while the molecular coevolutionary processes will be observable as they occur. Knowledge gained from this wealth of data could highlight the complex interaction between *Rafflesia* and its host, which in turn will be crucial to preserving the diversity and population

of this enigmatic plant. Information about the habitat preferences, host specificity and reproductive biology of the species will be helpful in biodiversity and conservation by revealing the environmental conditions that the populations require to survive.

Data Availability

No new datasets were generated or analyzed in this study.

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Disclosures

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