



Elucidating the evolutionary history of the Southeast Asian, holoparasitic, giant-flowered Rafflesiaceae: Pliocene vicariance, morphological convergence and character displacement

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ABSTRACT

The aim of the present study is to elucidate the evolutionary history of the enigmatic holoparasitic Rafflesiaceae. More specifically, floral morphological evolution is interpreted in a molecular phylogenetic context, the biogeographic history of the family is investigated, and the possibility of character displacement to have been operating in this family is assessed. Parsimony and Bayesian methods are used to estimate phylogeny and divergence times among Rafflesiaceae species based on nuclear and mitochondrial DNA sequence data from Barkman *et al.* (2008) as well as new sequence data from additional samples and an additional genetic marker, the plastid 16S. Ancestral areas are inferred using dispersal–vicariance analysis (DIVA) as well as a more recently developed parametric likelihood method (LAGRANGE), now including an update that allows for estimation over the posterior distribution of dated trees. Our extended molecular phylogeny of Rafflesiaceae implies a general lack of morphological synapomorphies as well as a high level of morphological homoplasy. In particular, a high level of floral morphological homoplasy is detected among *Rafflesia* species suggestive of similar patterns of pollinator-based selection in different geographic areas, and multiple instances of divergent floral size evolution is consistent with a model of character displacement. Initial diversification of Rafflesiaceae during the Late Cretaceous was followed by a long period of no-net diversification, likely due to extinctions caused by a Late Eocene to Miocene dramatic reduction in rainforest cover. A Late Miocene to Early Pliocene rise in sea-level probably caused the vicariant diversification observed between areas of endemism. The most recent species divergences are concordant with Pleistocene changes in climate and sea-levels, but apparently with no successful inter-area migrations, supportive of savannah, rather than rainforest, covered landbridges. An explosive increase in net diversification rate, most pronounced in *Rafflesia*, may be explained by Mid-Miocene to Pliocene rainforest-favorable conditions as well as natural selection promoting character displacement for *Rafflesia* flower size.

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1. Introduction

Rafflesiaceae is a family of holoparasitic flowering plants, perhaps most famous for comprising the world's largest single flower, *Rafflesia arnoldii*. As for all holoparasitic plants, Rafflesiaceae rely upon their host plant for both water and nutrients (Kuijt, 1969); however, they are unusual in that they are endoparasites emerging from the host only as ephemeral flowers during sexual

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reproduction. Rafflesiaceae lack vegetative parts and chlorophyll and grow as strands of cells embedded within the stem and root tissues of their host, woody climbers of the genus *Tetrastigma* (Miq.) Planch. (Vitaceae).

Morphological reductions, convergence and a highly divergent plant genome have long confounded the phylogenetic placement of Rafflesiaceae among the dicotyledonous angiosperms (Nickrent, 2002; Nickrent et al., 1998). Barkman et al. (2004) were the first to convincingly place Rafflesiaceae within the angiosperm order Malpighiales, and more recently the family was shown to be nested within the malpighialean family Euphorbiaceae (Davis et al., 2007) and to be sister to Euphorbiaceae s.s. (Wurdack and Davis, 2009). Molecular data also showed that Rafflesiaceae, as traditionally circumscribed, including nine genera of holoparasitic flowering plants (e.g. Takhtajan, 1997), is highly polyphyletic and that the three genera *Rafflesia*, *Rhizanthus* and *Sapria* form a natural group that is not closely related to any of the remaining six genera (Barkman et al., 2007; Barkman et al., 2004; Nickrent et al., 2004). Indeed, morphological studies had already pointed towards a close relationship between *Rafflesia*, *Rhizanthus* and *Sapria* (Beaman et al., 1992) that now constitute Rafflesiaceae sensu stricto (referred to as Rafflesiaceae hereafter). See Fig. 1 for a morphological comparison of these three genera.

While a placement within Malpighiales has become well-documented, inter-specific relationships within Rafflesiaceae have been less thoroughly investigated (but see Barkman et al., 2008). Despite extensive studies of morphological features of Rafflesiaceae species from the field, no phylogenetic analysis based on morphological characters has been published. A side-by-side study of the various species is difficult to achieve, as these plants have shown difficulty to cultivate and preserve. Several of the diagnostic characters of these giant fleshy flowers disappear (color) or change (size) with traditional preservation. Furthermore, the old herbarium specimens are often in bad condition (Bendiksby pers. obs.) and no single herbarium has specimens of all (or even most) species. Under these circumstances, molecular data are of utmost utility and the first molecular phylogeny of *Rafflesia* was recently published by Barkman et al. (2008). That study focused on rapid evolutionary flower size change in *Rafflesia* and revealed that although the three genera diverged from each other long ago, during the Late Cretaceous, most species have evolved quite recently, during the Pliocene to Pleistocene.

Rafflesiaceae are restricted to tropical rainforests of Southeast Asia and occur exclusively to the west of Wallace's line (Fig. 2a). The three species of *Sapria* occur in mountain forests in the seasonal climates of continental Southeast Asia, from India (Assam) to Thailand (Bänziger and Hansen, 1997; Elliott, 1990; Hansen, 1972) and do not overlap in distributional range with *Rafflesia* or *Rhizanthus* (Fig. 2a and d). *Rafflesia* and *Rhizanthus* occur in the more consistently wet forests of Western Malesia (Fig. 2a). The four *Rhizanthus* species occur on the Malay Peninsula, Sumatra, Java and Borneo, collectively called Sundaland and often grow in sympatry with *Rafflesia* (Fig. 2a and c). The more than 20 species of *Rafflesia* are patchily distributed from the Kra Isthmus Mountains in the southernmost part of Thailand, throughout Sundaland to the Philippines (Fig. 2a and b; Barcelona et al., 2009b; Meijer, 1997; Nais, 2001). Historical geological and climatic processes in Southeast Asia have undoubtedly exerted a strong influence on the phylogeny and biogeography of Rafflesiaceae. Although terrains corresponding to today's continental Southeast Asia and Sundaland have constituted one continuous area since the Jurassic, about 150 MyBP (Metcalfe, 1998: p. 38), there have been dramatic oscillations in rainforest cover and sea-level throughout the Cenozoic (Morley, 2007). Sundaland lies on Southeast Asia's relatively shallow continental shelf, the Sunda Shelf, which is bordered by deep sea, and during periods of low sea-level, islands on the Sunda

Shelf were linked by corridors of land to each other and to continental Asia (Hall, 1998). The most dramatic sea-level changes in Southeast Asia occurred during the Miocene and Pliocene (c. 23–2 MyBP). Relative sea-level during these epochs was at times considerably lower than at any time during the Pleistocene (e.g. Batchelor, 1979), and during the Late Pliocene, the sea may have flooded a larger portion of Sundaland than is the case at present (Turchyn and Schrag, 2004). However, Pleistocene sea-level fluctuations associated with glacial intervals have been considered the major factor in the development of biogeographic patterns in Sundaland (Moss and Wilson, 1998).

The phylogeny of Rafflesiaceae has also likely been influenced by biotic interactions with their seed dispersal agents (most likely rodents), hosts (about 10 species of *Tetrastigma*), and particularly their pollinators. The pollination syndrome of the entire family appears to be sapromyophily (Beaman et al., 1988; Bänziger, 1991, 1996, 2001, 2004; Bänziger and Hansen, 1997; Bänziger and Pape, 2004). One intriguing result of a floral size evolution study (Barkman et al., 2008) was that rapid floral size increases in *Rafflesia* are rivaled by rapid floral size decreases. Specifically, it appears that there have been high and concomitant rates of floral size increase and decrease in sister species descended from intermediate-sized ancestors in four separate geographic areas: Sumatra, Java, Peninsular Malaysia, and Borneo (Barkman et al., 2008: Fig. 2). Although the confidence intervals for ancestral flower size were wide, Barkman et al. (2008: Fig. S2) showed that there is only a small probability that the ancestors of *Rafflesia* species pairs were large making it unlikely that repeated instances of dwarfism (Davis, 2008) have occurred. However, as with any estimates of ancestral states, uncertainty makes directionality of floral size evolution changes challenging to discern. Assuming the ancestral state estimates were robust, it was hypothesized that their apparent opposing size changes were the result of character displacement because the divergent species pairs are sympatric in their current ranges (Barkman et al., 2008: Fig. 2). Character displacement in *Rafflesia* could arise from natural selection promoting the evolution of floral dimensions away from an intermediate size to avoid gamete wastage due to inter-specific hybridization (Armbruster et al., 1994; Grant, 1977; Muchhala and Potts, 2007). This process could promote, or at least maintain, reproductive isolation of sympatric species between which full intersterility has not yet evolved. Rafflesiaceae species attract a similar suite of sapromyophilous pollinators (Beaman et al., 1988; Bänziger, 2004; Bänziger and Hansen, 2000), and this sharing of pollen vectors could result in hybridization if the insects were not capable of distinguishing between congeners. Given that some of the same species of pollinator have been reported to visit different species and even genera of Rafflesiaceae, both large and small (Beaman et al., 1988; Bänziger, 2004; Bänziger and Hansen, 2000), mechanical reproductive isolation would be one way to prevent cross species fertilization by non-discriminating flies (Muchhala and Potts, 2007). Indeed, it appears that mechanical isolation may be achieved by different sized *Rafflesia* species because observations indicate that small-bodied pollinators do not acquire pollen from large flowered species (Beaman et al., 1988; Bänziger and Pape, 2004) and thus would be unlikely to effect pollination in such cases.

In the present study, we have continued our investigation of phylogenetic relationships in Rafflesiaceae by including more samples as well as an additional genetic marker (the plastid ribosomal small subunit, 16S). The phylogenetic results provide a context within which we first interpret the gross morphological patterns of evolution in the family. Secondly, by dating cladogenic events and inferring ancestral areas, we attempt to reconstruct the biogeographic history of Rafflesiaceae. Finally, we use a model-fitting approach to assess levels of support for an adaptive model of

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