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

Mika Bendiksby [a,b,*], Trond Schumacher [b], Galina Gussarova [a,h], Jamili Nais [c], Kamarudin Mat-Salleh [d,†], Nery Sofiyanti [d], Domingo Madulid [e], Stephen A. Smith [f], Todd Barkman [g]

[a] National Centre for Biosystematics, Natural History Museum, University of Oslo, P.O. Box 1172 Blindern, NO-0318 Oslo, Norway
[b] Department of Biology, University of Oslo, P.O. Box 1066, Blindern, NO-0316 Oslo, Norway
[c] Sabah Parks, 88806 Kota Kinabalu, Sabah, Malaysia
[d] School of Environmental and Natural Resources Science, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[e] Botany Division, Philippine National Museum, Burgos St., P.O. Box 2659, Manila, Philippines
[f] Department of Ecology and Evolutionary Biology, Brown University, Providence, RI 02912, USA
[g] Department of Biological Sciences, Western Michigan University, Kalamazoo, MI 49008, USA
[h] Department of Botany, St. Petersburg State University, Universitetskaya Nab. 7/9, St. Petersburg 199034, Russia

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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 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.

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

* Corresponding author at: National Centre for Biosystematics, Natural History Museum, University of Oslo, P.O. Box 1172, Blindern, NO-0318 Oslo, Norway. Fax: +47 22 85 18 35.
E-mail address: annamb@nhm.uio.no (M. Bendiksby).
† Deceased.

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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*, *Rhizanthes* 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*, *Rhizanthes* 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 difficult 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 *Rhizanthes* (Fig. 2a and d). *Rafflesia* and *Rhizanthes* occur in the more consistently wet forests of Western Malesia (Fig. 2a). The four *Rhizanthes* 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

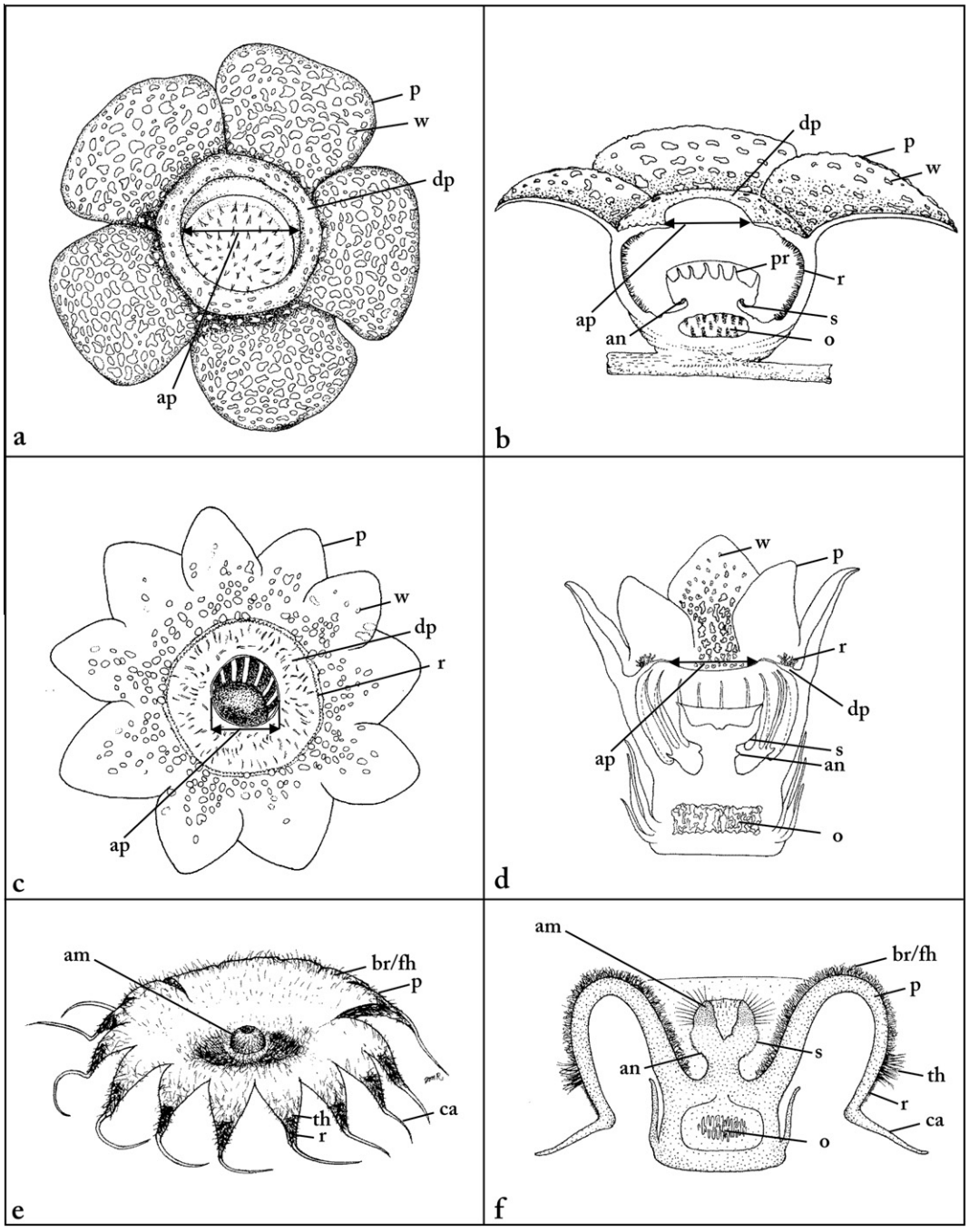

Fig. 1. Drawings of female flowers from above (left) and in cross-section (right) of *Rafflesia* (a and b; after Figs. 1 and 12 in Nais 2001), *Sapria* (c and d; after Figs. 1 and 11 in Bänziger and Hansen, 1997) and *Rhizanthes* (e and f; after Figs. 17 and 25 in Bänziger and Hansen, 2000), with selected morphological terms: ap = aperture, am = ampulla, an = anthers (position in male flowers), br = bristles, ca = caudal appendage, dp = diaphragma, fh = furry hairs, p = periogone lobe (called tepal in *Rhizanthes*), pr = processes, r = ramenta, s = stigmatic area, th = tuft hairs, and w = wart. Drawings by Jon Reierstad (Faculty of Mathematics and Natural Sciences, Section for Photo and Grafics, University of Oslo).

character displacement relative to a non-adaptive model to describe patterns of floral size evolution.

2. Materials and methods

2.1. Taxon sampling and molecular methods

The present study is based on DNA sequence data from Barkman et al. (2008), 24 newly generated sequences and a few additional sequences obtained from GenBank. About 80% of Rafflesiaceae species, including all three genera, covering most of the family's geographic distribution, are represented with DNA sequence data from all three genomic compartments (the plastid ribosomal 16S gene, the mitochondrial *mat*R and *atp*6 genes and the *nad*1 B–C intron, and the nuclear ribosomal ITS region). For phylogenetic rooting and dating purposes, two malpighialean taxa were included, *Neoscortechinia kingii* Pax and K. Hoffm. (Euphorbiaceae s.s.) and *Ixonanthes chinensis* Champ. (Ixonanthaceae).

Vouchering of Rafflesiaceae material is difficult, and it is not always possible to deposit specimens as outlined in Pleijel et al. (2008). The species are protected by law, and only with special permission can small pieces of tissue be collected. Due to the

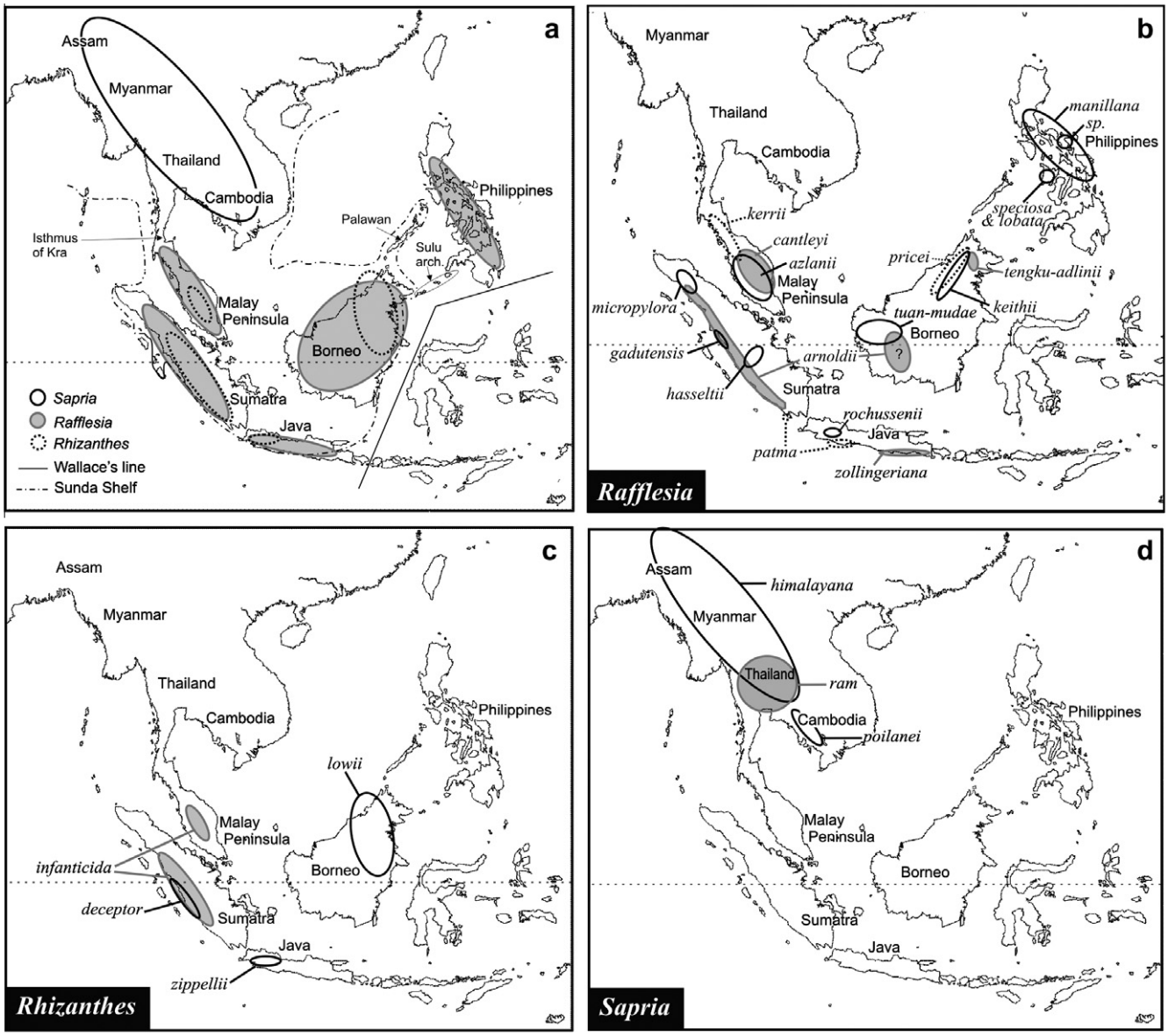

Fig. 2. Maps of Southeast Asia with distributions of Rafflesiaceae genera and species. Wallace's line is indicated. (a) Distributional map of the three Rafflesiaceae genera: *Rafflesia*, *Rhizanthes* and *Sapria*. (b) Distributional map of *Rafflesia* species. (c) Distributional map of *Rhizanthes* species. (d) Distributional map of *Sapria* species.

sporadic nature of blooming and high rates of bud mortality it is difficult to predict when mature flowers will be available for collection. Therefore, when buds were sampled they were collected from historically and continuously monitored sites known to have only one species of *Rafflesia* or *Rhizanthes* occurring there. When flowers were present, detailed photographs were taken and stored together with preserved tissue material as vouchers. DNA was extracted, amplified and sequenced as described in Barkman et al. (2008). The forward primer Raff16SF (5′ GCTGGAGTACGGT AGGGGCAG 3′) and the reverse primer Raff16SR (5′ ACGAGGGTTG CGCTCGTTGC 3′) were used for both PCR and sequencing of the plastid 16S. New sequences have been submitted to GenBank, and voucher information and GenBank accession numbers are provided in Table 1.

2.2. Alignment and phylogeny reconstruction

DNA sequences were assembled and edited using Sequencher™ 4.1.4 (© 1991–2002 Gene Codes Corporation) and manually aligned using BioEdit version 7.0.9.0. (Hall, 1999). Maximum Parsimony (MP) and Bayesian inference (BI) analyses were performed on each genetic region separately and in combination.

Maximum parsimony trees were reconstructed using PAUP* 4.0b10 (Swofford, 2003) with all characters treated as unordered and having equal weights. Gaps were treated as missing and ambiguities as uncertainty. Starting trees were obtained via stepwise addition (random sequence addition). Heuristic searches were conducted with 2000 replicates and 10 trees held at each step. The tree-bisection-reconnection (TBR) algorithm was used for branch-swapping, and the steepest descent option was not in effect. Branches were collapsed if minimum branch lengths were zero, and the 'MulTrees' option was in effect.

For Bayesian inference (BI) analyses and divergence time estimation (see below) we determined the optimal substitution model for the various genetic regions using the Akaike Information Criterion (AIC) in MrModeltest (Nylander, 2004). Because the same model was selected for all genetic regions (GTR + G), our analyses have been performed on an unpartitioned concatenated matrix. Bayesian inference analyses were conducted using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), twice, each with four chains.

Table 1
List of species names, voucher information and GenBank accession numbers for sequences of specimens used in this study.

Species name	Voucher No. (Herb)	GenBank accession No.				
		*mat*R	*nad*1 B–C	*atp*6	ITS	16S
Ixonanthes chinensis Champ.	Chen 9812087 (?)	AY674526	N/A	HM803246	N/A	N/A
Neoscortechinia kingii Pax & K. Hoffm.	Chase 1265 (K)	AY674543	N/A	N/A	N/A	N/A
Rafflesia arnoldii R.Br.	MB 612.2 (O)	EU882311	EU882330	EU882274	EU882292	HM803255
Rafflesia azlanii Latiff & M. Wong	K. Mat-Salleh s. n. 21 Aug. 2003 (UKMB)	HM803248	HM803269	HM803245	HM803265	N/A
Rafflesia cantleyi Solms	K Mat-Salleh et al. CJR1 f5 (UKMB)	EU882314	EU882333	EU882277	EU882295	HM803258
Rafflesia gadutensis Meijer	MB 620.1 (O)	EU882309	EU882328	EU882272	EU882290	HM803253
Rafflesia hasseltii Suringar	MB 637.2 (O)	EU882310	EU882329	EU882273	EU882291	HM803254
Rafflesia keithii Meijer	TJB 402 (SNP)	AY453074	EU882332	EU882276	EU882294	HM803257
Rafflesia kerrii Meijer	K. Mat-Salleh s. n. 2 Jan. 2003 (UKMB)	EU882315	EU882334	EU882278	EU882296	HM803259
Rafflesia lobata R. Galang & Madulid	Galang et al. 001 (PNH)	N/A	N/A	N/A	HM803267	N/A
Rafflesia manillana Teschem.	E. S. Fernando s. n. January 2005	EU882319	N/A	EU882282	EU882300	N/A
Rafflesia micropylora Meijer	MB 904.1 (O)	EU882316	EU882335	EU882279	EU882297	HM803260
Rafflesia patma Blume.	MB 404 (O)	EU882307	EU882326	EU882270	EU882288	HM803251
Rafflesia pricei Meijer	CWD 99.01 (SNP)	EU882312	EU882331	EU882275	EU882293	HM803256
Rafflesia rochussenii Teijsm. & Binn.	MB 700.3 (O)	EU882306	EU882325	EU882269	EU882287	HM803250
Rafflesia sp.	Jaucian-Adan and Valenzuela 101 (PNH)	HM803249	HM803270	N/A	HM803266	HM803263
Rafflesia speciosa Barcelona & Fernando	Ferdinand Gaerlan No.29001 (PNH)	EU882320	N/A	N/A	EU882301	N/A
Rafflesia tengku-adlinii Mat-Salleh & Latiff	KNP a15095 (SNP)	EU882317	EU882336	EU882280	EU882298	HM803261
Rafflesia tuan-mudae Becc.	K. Mat-Salleh & C. Ko, KMS5385 (UKMB)	EU882318	EU882337	EU882281	EU882299	HM803262
Rafflesia zollingeriana Koord.	MB 603.3 (O)	EU882308	EU882327	EU882271	EU882289	HM803252
Rhizanthes deceptor H. Bänziger & B. Hansen	MB 619.2 (O)	EU882323	EU882340	EU882285	EU882304	N/A
Rhizanthes infanticida H. Bänziger & B. Hansen	MB 608.2 (O)	EU882322	EU882339	EU882284	EU882303	N/A
	Nickrent 2844 (?)	AY739010	N/A	N/A	N/A	N/A
Rhizanthes lowii (Becc.) Harms	SNP14705 (SNP)	AY453073	EU882338	EU882283	EU882302	HM803264
Sapria himalayana Griff.	Chayan & CK Lim, L7966 (UKMB)	HM803247	HM803268	EU882268	EU882286	N/A
	Nickrent 4156 (?)	AY739006	N/A	N/A	N/A	N/A
	Qiu99-028 (?)	AY674561	AY674768	N/A	N/A	N/A
Sapria poilanei Gagnep.	AB142 (?)	AY739004	N/A	N/A	N/A	N/A
Sapria ram H. Bänziger and B. Hansen	AB129 (?)	AY739005	N/A	N/A	N/A	N/A

Each analysis was run for 2 million steps, after starting from a random tree, sampling every 1000 generations. The model parameters in the Bayesian analyses were treated as unknown variables with default prior probability distributions. To test whether the Markov Chain converged, we monitored the standard deviation of split frequencies (SDSF), which did fall below 0.01 (0.0064 at termination) when comparing two independent runs. The first 500 steps were discarded as burn-in, the point when the SDSF permanently fell below 0.01. The results of the analysis were summarized as a 50% majority rule consensus tree.

Branch support was obtained by both parsimony and likelihood bootstrapping with 2000 and 100 replicates of full heuristic searches, respectively. Maximum likelihood analyses were performed using the same model of nucleotide substitution as chosen for the BI analyses using PAUP* 4.0b10. Although a heuristic analysis using maximum likelihood was performed, the results are not presented below because they did not differ qualitatively from the parsimony and BI results.

2.3. Divergence time estimation

Because the same substitution model was selected for all included genetic regions, a concatenated unpartitioned data set of five genetic markers from three genomic compartments with a malpighialean outgroup was used for estimating the posterior probability distribution of divergence times in Rafflesiaceae using the computer program Bayesian Evolutionary Analysis Sampling Trees (BEAST) 1.4.7 (Drummond and Rambaut, 2007). This approach co-estimates phylogeny and divergence dates using strict- or relaxed molecular clock models (Drummond et al., 2006).

Since there are no known fossils of Rafflesiaceae, we used secondary (indirect) calibration points, i.e. divergence time estimates obtained from a different dataset on the basis of primary calibration points (fossils), and this could be a potential source of error in our analyses. The crown group age of Malpighiales has been estimated by various authors (e.g. Davis et al., 2005; Magallon and Castillo, 2009; Wang et al., 2009; Wikström et al., 2001) ranging from 77 My (Wikström et al., 2001) to about 115 My (Davis et al., 2005). Therefore, two malpighialean species were included for calibration purposes: *Neoscortechinia kingii* (Euphorbiaceae s.s.) and *Ixonanthes chinensis* (Ixonanthaceae). Initial divergence of Malpighiales appears to have occurred over a very short time period (Davis et al., 2005) justifying the use of the crown group age of Malpighiales for these analyses.

BEAUti v. 1.5.4 (Drummond and Rambaut, 2007) was used to produce the script (xml-file) for BEAST. The prior for the time to the most recent common ancestor of Rafflesiaceae plus *Neoscortechinia* (i.e. all but *Ixonanthes*) was set to a lognormal distribution with log mean = 2.9 and lognormal standard deviation = 0.4 and offset set to 77 My, i.e. the youngest of several published age estimates for Malpighiales (Wikström et al., 2001). A lognormal distribution for priors fixes the minimum age of a calibrated node and allows the maximum age to be sampled following a lognormal distribution with no hard limit (Ho and Phillips, 2009). As BEAST may get stuck on local optima because it does not employ a coupled MCMC, monophyletic sets of taxa (nodes 1, 5, 13 and 19) were predefined in order to obtain a topology that corresponds to the phylogeny obtained from the more robust phylogenetic methods (e.g. MrBayes). A Yule tree prior was employed in all runs, which assumes a constant speciation rate for each branch in the tree. The defaults in BEAUti were used for all other parameters. The Markov chains were run for 10 million generations, sampling and saving every 1000th tree, in order to obtain effective sample sizes of more than 200 for all parameters. Convergence of the chain to stationary distributions was confirmed by inspection of the MCMC samples in each analysis using the program Tracer 1.4 (Rambaut and Drummond, 2007). The BEAST analysis was performed twice (to determine if the two independent runs converged on the same posterior distribution) and log output files were compared using Tracer. Analyses were also run without data in order to sample from the joint prior distribution only.

Shifts in birth and death rates can leave distinctive signatures in phylogenies, resulting in departures from linearity in semi-log lineage through time (LTT) plots (Nee, 2006). Based on the obtained phylogeny and divergence time estimates, a plot of the natural logarithm trace of lineage accumulation through time was made using the computer software RGui (R Developmental Core Team, 2009), and this was compared to theoretical LTT plots in Crisp and Cook (2009: Fig. 1). Outgroups were excluded to avoid artifacts resulting from undersampling of species (Nee et al., 1995).

2.4. Ancestral area analyses

A number of methods have been proposed to recognize unit areas that are appropriate for the level of study (e.g. Axelius, 1991; Harold and Mooi, 1994; Humphries and Parenti, 1986; Morrone, 1994). We predefined unit areas based on geological information (Hall, 1998) and the present-day Rafflesiaceae distributions (Fig. 2a–d). Locality information was obtained from herbarium specimens and supplemented with information from the literature (Bänziger and Hansen, 1997, 2000; Nais, 2001). Six unit areas (A–F) were recognized: A. Assam and central and northern parts of Myanmar; B. Thailand north of Kra Isthmus (hereafter referred to as Thailand); C. Borneo; D. The Malay Peninsula and Thailand south of Kra Isthmus (hereafter referred to as the Malay Peninsula); E. Sumatra and Java (collectively referred to as Indonesia); and, F. the Philippines. Each terminal was coded for the unit area(s) in which it presently occurs.

Ancestral areas were inferred using dispersal and vicariances analysis (Ronquist, 1997), as implemented in the computer program DIVA (Ronquist, 1996), and a parametric likelihood analysis with a dispersal–extinction–cladogenesis model (Ree et al., 2005) as implemented in the computer program LAGRANGE v. 2.0.1 (Ree and Smith, 2008). Whereas the vicariance-based approach in DIVA is derived from character optimization methods, where optimality is determined by a parsimony criterion (minimum overall dispersal and extinction costs), LAGRANGE enables maximum likelihood estimation of the ancestral states (range inheritance scenarios) at cladogenesis events by modeling transitions between discrete states (ranges) along phylogenetic branches as a function of time.

The phylogenetic relationships among species, including the location of the root, are assumed to be known and fully bifurcate in both DIVA and LAGRANGE. Therefore, the branching order of the fully resolved maximum sum of clade credibilities tree from the dating analysis was used for the dispersal–vicariance analyses of Rafflesiaceae taxa (no outgroup included). An inherent weakness of dispersal–vicariance analysis is that the number of ancestral areas increases at deeper nodes in the tree due to its assumption of vicariant evolution (Ronquist, 1997). We therefore explored different maxarea settings from six to two using the optimize command and otherwise used default option settings. In order to take into account phylogenetic and divergence time uncertainty in the historical biogeographic analysis, LAGRANGE was performed on 1000 trees randomly selected from the posterior distribution of dated trees, a procedure first used in Smith (2009), and herein performed using an updated approach (LAGRANGE C++) with considerably reduced computational time. Although connectivity between areas can be extensively modeled using LAGRANGE, we did not infer any constraints on movements between areas in our present analyses. *Neoscortechina* was coded for its center of diversity (BCDE) and *Ixonanthes* for all areas.

2.5. Character displacement model fitting

To examine whether the pattern of floral size variation among species is consistent with character displacement we used OUCH (Butler and King, 2004) to compare the non-adaptive Brownian motion model to the adaptive Ornstein–Uhlenbeck model (Hansen, 1997). For the adaptive model of character displacement, selective optima were assigned to branches on the dated *Rafflesia* phylogeny obtained in this study (see Results section). All internal branches were assigned to an unknown ancestral floral size selective optimum while those at the tips were assigned an intermediate floral size if the species is currently non-geographically overlapping with any other species. For those that have overlapping geographic distributions, we assigned a large floral size selective optimum to the larger sympatric species, and a small floral size selective optimum to the smaller of the sympatric species. Floral sizes assigned to extant taxa were taken from Barkman et al. (2008) and original descriptions. This analysis scored all species at their floral size-range minima. In a second more conservative analysis, we scored all taxa for their floral size minima except for the sympatric ones which were scored at the size range minimum if the larger of a pair and at their size range maximum if the smaller of a pair. Models were compared by both AIC and LRT. We used the divergence times estimated herein (see Results section) to compare the fit of the two models. In addition, in order to evaluate the effect of phylogenetic uncertainty on the model-fitting procedure, we sampled 20 trees randomly from the posterior distribution of topologies and branch lengths generated by the BEAST analyses described above.

3. Results

3.1. Alignment and phylogeny reconstruction

Lengths of aligned genetic regions were as follows: *atp*6, 657 basepairs (bp); *mat*R, 1587 bp; *nad*1 B–C intron, 3117 bp; ITS, 739 bp; and 16S, 472 bp. Phylogenetic reconstructions/estimates from independent analyses of the different genetic regions were congruent but variously resolved; *atp*6 and 16S provided the least resolution and *nad*1 B–C intron provided the most (data not shown). The concatenated data set included 29 taxa and was 6572 nucleotides long, including gaps and missing data. The independent runs using MrBayes on the concatenated dataset converged to identical consensus topologies (Fig. 3) with some minor variation in the posterior probability values. Parsimony analysis resulted in 80 most parsimonious trees of length 864 steps. Although some branches were weakly supported by parsimony and likelihood bootstrapping, the level of homoplasy is low, as indicated by high consistency-, retention- and rescaled consistency indices (CI $\approx$ 0.94, RI $\approx$ 0.95 and RC $\approx$ 0.89, respectively). The concatenated matrix and phylogeny have been made available at TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S10732).

The family's three component genera form strongly supported monophyletic groups, and *Rafflesia* and *Rhizanthes* are sisters (Fig. 3), corroborating previous findings (e.g. Barkman et al., 2008; Nickrent et al., 2004; Wurdack and Davis, 2009). Indonesian, Bornean, Peninsular Malaysian and Philippine species of *Rafflesia* are reciprocally monophyletic, although the Bornean clade is not supported by bootstrapping above 50%. Patterns of relationships among some recently evolved species are less supported. Monophyly is strongly supported for species for which two or more accessions from different localities were analyzed (*Rhizanthes infanticida* and *Sapria himalayana* (Fig. 3), and for *Rafflesia arnoldi* and *Ra. gadutensis* [data not shown]). Also, *mat*R sequences of *Ra. keithii* (AY739007) and *Ra. pricei* (AY739008) in GenBank are identical to the sequences included for these species in the present study. Inter-specific sequence variation within each unit area is very low in *Rafflesia* (Fig. 3).

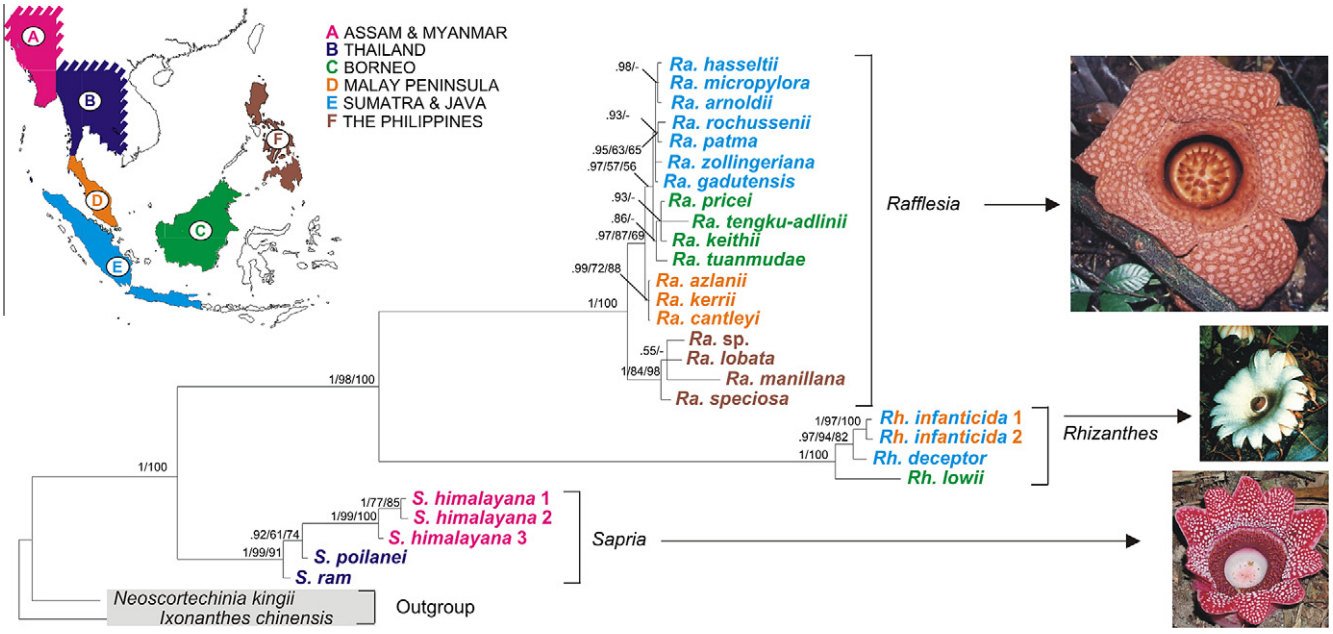

Fig. 3. Majority rule phylogram from the Bayesian inference analysis on the concatenated matrix. Bayesian posterior probability (PP) and both parsimony- (MP) and maximum likelyhood (ML) bootstrap values are reported above branches separated by slashes in order PP/MP/ML (ML bootstrap only reported when different from MP bootstrap). Colors on terminals refer to the colored areas of the inset map showing Southeast Asia with the six unit areas (A–F). *Rhizanthes infanticida* occur both on Peninsular Malaysia and in Indonesia. Malpighialean outgroup (*Neoscortechinia kingii* [Euphorbiaceae s.s.] and *Ixonanthes chinensis* [Ixonanthaceae]) in grey shading.

3.2. Divergence time estimation

The maximum sum of clade credibilities chronogram is presented in Fig. 4. Grey bars denote the 95% highest posterior density (HPD) intervals of the posterior probability distribution of node ages, and the asterisk indicates the calibrated node. Median node ages and the 95% HPD are summarized in Table 2. According to these results, the Rafflesiaceae stem group age is about 95 My old (node 1) and initial diversification (Rafflesiaceae crown group) is inferred to have occurred about 82 MyBP (node 2). After the *Rafflesia-Rhizanthes* split, about 73 MyBP (node 3), no further diversification is revealed until Late Miocene (12–5 MyBP) when initial intra-generic diversification occurred in all three genera (nodes 4, 5, 8, and 11–13). The first intra-area divergences happened during Late Miocene in Thailandic *Sapria* and Philippine *Rafflesia* species (nodes 4, 12 and 13). Subsequent inter- and intra-area diversifications occurred after the onset of the Pliocene (Fig. 4).

The corresponding lineage diversity through time (LTT) plot (Fig. 5a) shows a 60 My period of no net diversification from about 73 MyBP, followed by an explosive increase in net diversification rate beginning about 12 MyBP. The rate of diversification increased steadily through the Pliocene (5–2 MyBP) and Pleistocene (2–0.01 MyBP), mostly accounted for by diversification in *Rafflesia* and less by *Rhizanthes* and *Sapria* (Fig. 5b). This pattern of diversification through time produces an antisigmoid curve of the LTT plot (Fig. 5a), i.e. the line rises steeply to a plateau after which is rises steeply again, a shape retained also when confidence intervals are included (Fig. 5a).

3.3. Ancestral area analyses

Inferred ancestral areas at relevant nodes (nodes 2–27) from DIVA and LAGRANGE analyses are summarized in Table 2. Five nodes included alternative equally parsimonious solutions from DIVA (Fig. 4: nodes 2, 3, 4, 11 and 15), but for the great majority of nodes, only a single most parsimonious solution was reconstructed. Restricting the number of allowed ancestral areas mainly affected the deeper nodes and gradually increased the number of inferred dispersals from five to seven (Table 2). The proportions of summed proportional likelihoods of all inferred areas for each node from LAGRANGE analyses of 1000 dated trees are presented in Table 2 and superimposed on Fig. 4. After deleting results that were present in less than 5% of the analyses, only five nodes included uncertainty in inferred ancestral areas (Fig. 4: nodes 2, 3, 4, 8 and 9). Uncertainty was extensive for the oldest nodes in the tree (Fig. 4: nodes 0–3, 0 and 1 not shown), whereas all other nodes included only one or two alternatives (Fig. 4). Time of divergence did not seem to be a very influential parameter on ancestral area inference (not shown), and is therefore not emphasized herein. Inferred ancestral areas using the two different methods are highly congruent. Solutions obtained from LAGRANGE correspond to inferred areas by DIVA at all nodes except for three consecutive nodes in the *Rhizanthes* clade (Fig. 4: nodes 8, 9 and 10), for which LAGRANGE estimated larger areas. Area combinations that include the Philippines can be excluded for node two and three, as the Philippines had not become part of Southeast Asia at the time (Hall, 1998).

3.4. High rates of diversification in Rafflesia may be due to natural selection promoting character displacement

Because the rate of diversification in *Rafflesia* has increased dramatically in the Pliocene and Pleistocene (Fig. 5b), and because previous analyses indicated that currently sympatric species exhibit divergent floral diameters, which evolved from intermediate-sized ancestors (Barkman et al., 2008), we fitted a model of character displacement to the data. This explicit model-fitting procedure scoring all taxa at their floral size minima indicates that an adaptive model of character displacement is a significantly better fit to the data than one implicating a non-adaptive Brownian motion model (LRT = 21.8, d.f. = 5; $P < 0.001$; ΔAIC = 11.9). The second more conservative analysis also resulted in a significantly better fit of the adaptive model of character displacement as compared to the non-adaptive Brownian motion model (LRT = 11.4, d.f. = 5; $P = 0.04$; ΔAIC = 1.5). Statistical support for the adaptive model of floral size variation did not depend on topology when sizes were scored at size-range minima; however, using the conservative

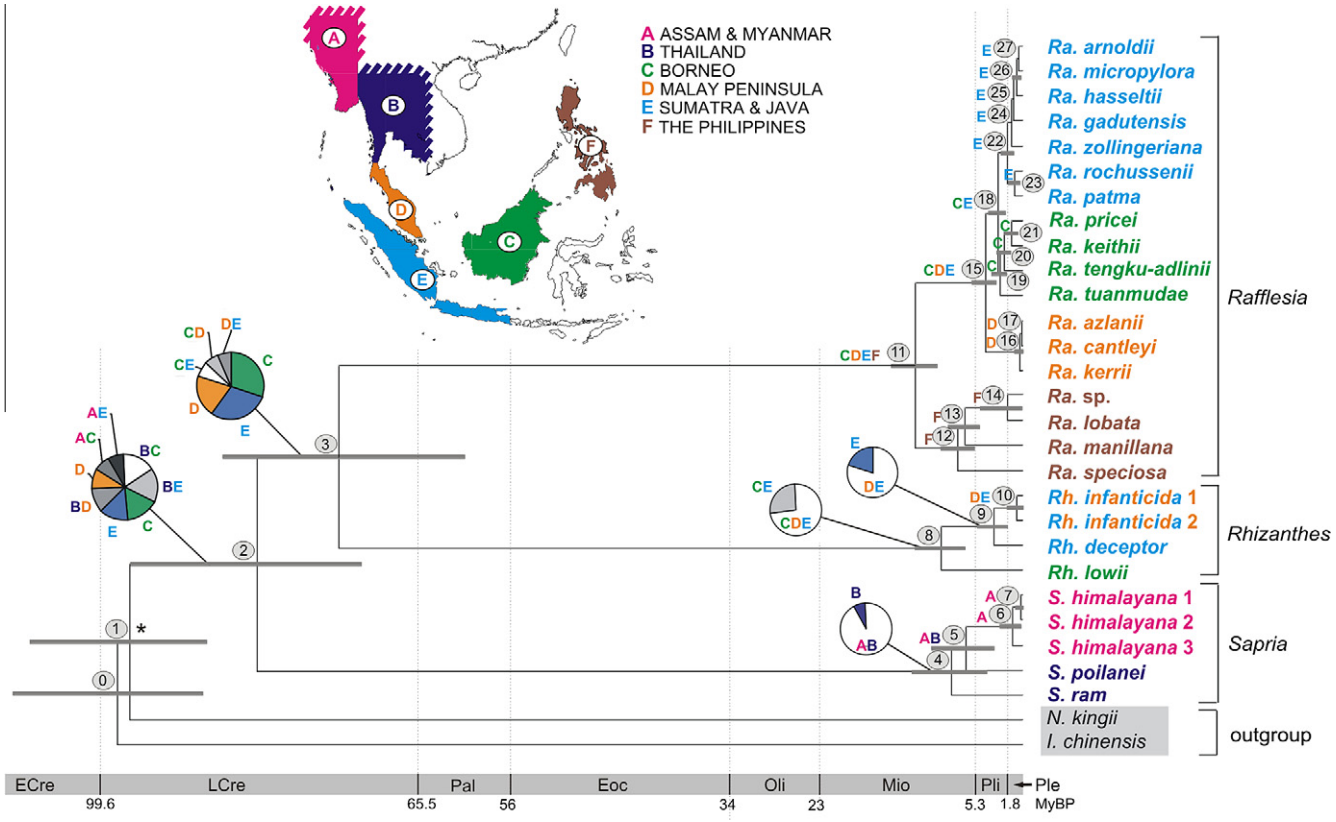

Fig. 4. Maximum sum of clade credibilities tree produced from divergence time analysis using BEAST. The calibrated node is indicated by an asterisk. Grey bars denote the 95% highest posterior density (HPD) intervals of the posterior probability distribution of node ages. Numbers on scale axis are in millions of years before present (MyBP). Colors on terminals refer to the colored areas of the inset map showing Southeast Asia with the six unit areas (A–F). Inferred ancestral areas for internal nodes obtained from LAGRANGE on 1000 trees sampled from the posterior distribution of dated trees are summarized and superimposed. Results represented in less that 5% of the analyses are not reported. See table 2 for more details from the ancestral area analyses.

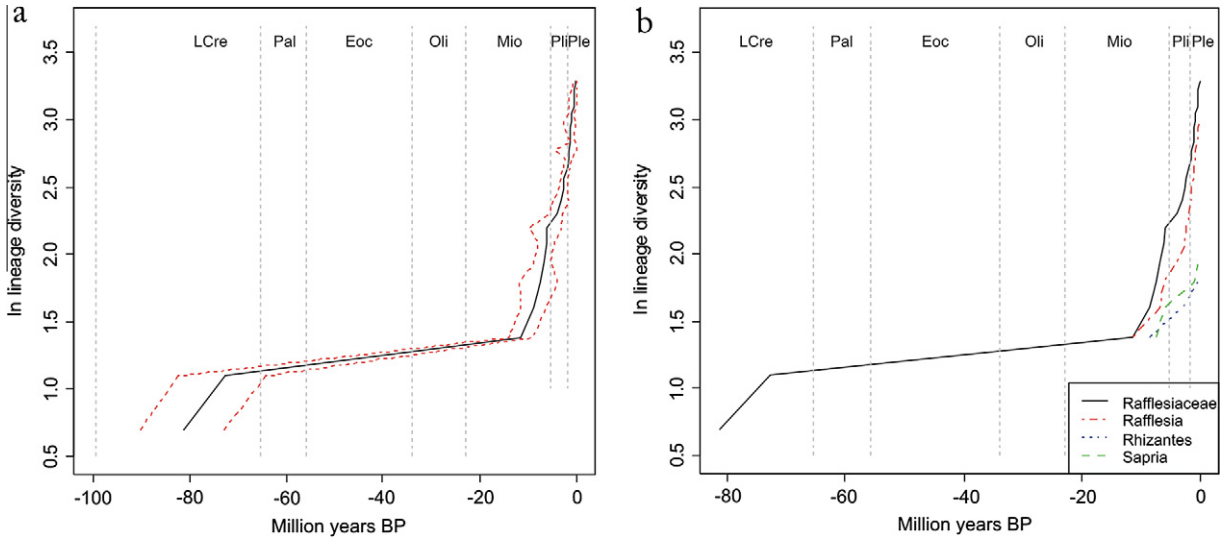

Fig. 5. Lineage through time (LTT) diversity plots. Numbers on the *X*-axis refer to time before present in million years. Numbers on the *Y*-axis is the natural logarithm of lineages diversity. (a) LTT diversity plot of Rafflesiaceae with the 95% HPD indicated with red stippled lines. (b) LTT plots of each of the three genera (*Rafflesia*, *Rhizanthes* and *Sapria*) shown simultaneously and indicated by variously colored and stippled lines. *Abbreviations*: ln = the natural logarithm, LCre = Late Cretaceous, Pal = Paleocene, Eoc = Eocene, Oli = Oligocene, Mio = Miocene, Pli = Pliocene and Ple = Pleistocene.

floral size scoring, $P > 0.05$ for ca. 20% of trees in the posterior probability distribution generated by BEAST.

Because the adaptive model with four selective optima is preferred for *Rafflesia*, we tested the predictions of the hypothesis of character displacement. If selection for size divergence to avoid gamete wastage has resulted in character displacement, we expected that pollen and stigma chambers, into which pollinators need to precisely fit to acquire and deposit pollen masses, respectively, would be divergent between sympatric congeners. Indeed, the dimensions of the pollen and stigmatic chambers, are, as

Table 2
Results from various analyses: (from left) Node numbers that refer to Fig. 4; Bayesian posterior probability and parsimony- and likelihood bootstrap support, respectively; node age estimates; 95% highest posterior density values (HPD) of age estimates (depicted as grey bars in Fig. 4); inferred ancestral areas at internal nodes using DIVA and for various maxarea settings; and, proportion of proportional likelihoods (in brackets) of inferred ancestral areas using LAGRANGE on 1000 trees from the posterior distribution of BEAST chronograms. Number of dispersals with varying maxarea settings (DIVA) are presented in the last row. Unit areas: A = Assam and Myanmar, B = Thailand north of Kra Isthmus, C = Borneo, D = Malay Peninsula and Thailand south of Kra Isthmus, E = Sumatra and Java, F = The Philippines.

Node	Branch support	Node age	95% HPD	DIVA					LAGRANGE C++
				Unrestricted	Maxareas = 5	Maxareas = 4	Maxareas = 3	Maxareas = 2	
0	N/A	96.14	[83.595, 110.73]	N/A	N/A	N/A	N/A	N/A	N/A
1	N/A	95.02	[83.14, 109.47]	N/A	N/A	N/A	N/A	N/A	N/A
2	1/100/100	81.67	[69.47, 95.89]	BCDEF, ABCDEF	BCDEF	BCDE, BCDF, BCEF, BDEF	BC, ABC, BCD, BE, ABE, BCE, BDE, BCF, BDF, BEF	BC, BE	BC (0.138), BE (0.129), C (0.121), E (0.11), BD (0.096), D (0.084), AC (0.068), AE (0.063)
3	1/98/100	73.19	[60.76, 86.63]	CDEF	CDEF	CDE, CDF, CEF, DEF, CDEF	C, CD, E, CE, DE, CDE, CF, CDF, EF, CEF, DEF	C, E	C (0.299), E (0.279), D (0.2), CE (0.08), CD (0.065), DE (0.06)
4	1/99/91	8.65	[4.56, 13.61]	B, AB	B	B	B, AB	B	AB (0.924), B (0.075)
5	.92/61/74	6.98	[3.45, 11.08]	AB	AB	AB	AB	AB	AB (1)
6	1/99/100	1.43	[0.26, 2.88]	A	A	A	A	A	A (1)
7	1/77/85	0.29	[0, 0.88]	A	A	A	A	A	A (1)
8	1/100/100	9.07	[6.37, 12.3]	CE	CE	CE	CE	CE	CDE (0.722), CE (0.276)
9	.97/94/82	3.33	[1.67, 5.08]	E	E	E	E	E	DE (0.79), E (0.209)
10	1/97/100	0.79	[0.03, 1,8]	E	E	E	E	E	DE (1)
11	1/100/100	11.82	[9.23, 15.06]	DF, CDF, DEF, CDEF	DF, CDF, DEF CDEF	CF, DF, CDF, EF, CEF, DEF, CDEF	CF, DF, CDF, EF, CEF, DEF	CF, EF	CDEF (1)
12	1/84/98	7.17	[5.2, 9.31]	F	F	F	F	F	F (1)
13	.55/–/–	6.46	[4.6, 8.43]	F	F	F	F	F	F (1)
14	–/–/–	2.34	[0.07, 5.11]	F	F	F	F	F	F (1)
15	.97/87/69	4.25	[2.93, 5.79]	CD, DE, CDE	CD, DE, CDE	CD, DE, CDE	CD, DE, CDE	CD, DE	CDE (1)
16	.99/72/88	0.38	[0.02, 0.94]	D	D	D	D	D	D (1)
17	–/–/–	0.13	N/A	D	D	D	D	D	D (1)
18	.97/57/56	2.89	[1.94, 3.9]	CE	CE	CE	CE	CE	CE (1)
19	.86/–/–	2.66	[1.75, 3.6]	C	C	C	C	C	C (1)
20	.93/–/–	2.13	[1.35, 2.96]	C	C	C	C	C	C (1)
21	–/–/–	1.32	[0.53, 2.18]	C	C	C	C	C	C (1)
22	.93/–/–	1.77	[0.99, 2.6]	E	E	E	E	E	E (1)
23	.95/63	1.01	[0.36, 1.72]	E	E	E	E	E	E (1)
24	–/–/–	1.35	N/A	E	E	E	E	E	E (1)
25	–/–/–	1.05	N/A	E	E	E	E	E	E (1)
26	.98/–/–	0.68	[0.21, 1.22]	E	E	E	E	E	E (1)
27	–/–/–	0.43	N/A	E	E	E	E	E	E (1)
Number of inferred dispersals				5	5	6	7	7	

predicted, larger in *Ra. kerrii* (8 and 6.5 mm, respectively; $n = 4$ for both) as compared to the sympatric, smaller, close relative, *Ra. cantleyi* (4 and 4.5 mm, respectively; $n = 8$ and 5, respectively). Another prediction resulting from the hypothesis of selection for character displacement is that floral morphological differences will be more pronounced in sympatry as opposed to allopatry (Grant, 1977). In Peninsular Malaysia, the diameter of *Ra. cantleyi* is 31 cm on average in regions of overlap with *Ra. kerrii* whereas it is ca. 41 cm on average to the south where the two are allopatric ($t = -3.64$, d.f. = 27, n = 43, $P < 0.001$). Sample sizes are small for *Ra. kerrii*, but reports from Thailand range from 50–90 cm while in Peninsular Malaysia, in the broad region of overlap with *Ra. cantleyi*, floral size ranges from 70–100 cm ($n = 11$).

4. Discussion

4.1. General

This study builds upon our previous molecular phylogenetic analysis of Rafflesiaceae inter-specific relationships (Barkman et al., 2008) to which we have added more samples and an additional genetic marker, the plastid 16S ribosomal region. While we have not demonstrated that the 16S sequences are encoded by the plastid genome of Rafflesiaceae, they are homologous to those of other angiosperms and other extreme holoparasites appear to retain plastid encoded 16S (Nickrent et al., 1997). Future studies could aim to look at hybridization intensity on Southern blots to discern whether these 16S sequences are organellar or nuclear encoded. To distinguish if the sequences are plastid as opposed to transferred to the mitochondrion may require genomic sequencing. If they are encoded by a plastid genome in these species, the results would be the first evidence for the existence of a plastid genome in Rafflesiaceae and the phylogenetic results presented should be reliable because they would reflect variation in all three genomic compartments.

Holoparasitic angiosperms are well-known for their high rates of nucleotide substitution (Nickrent and Starr, 1994) and long branches in angiosperm phylogeny (Nickrent et al., 2004). From this, and from molecular phylogenetic works of inter-species relationships of other holoparasitic angiosperms (e.g. Nickrent et al., 1994), we had expected to see long branches also at the inter-species level in Rafflesiaceae. The short-branched inter-specific relationships, especially within *Rafflesia*, were therefore unexpected (Barkman et al., 2008), not the least given the large variation in some morphological characters such as floral size, floral shape, pattern and number of white warts, length and shape of ramenta, number of processes and size of diaphragm orifice (see Nais, 2001). Despite low levels of molecular divergence in *Rafflesia*, phylogenetic relationships that were weakly supported in Barkman et al. (2008) are retained when more taxa and sequences have been included – the only differences being monophyly of the Bornean *Rafflesia* species and the phylogenetic sister-relationship between the Indonesian and Bornean *Rafflesia* clades (Fig. 3). In Barkman et al. (2008), Bornean *Rafflesia* species constituted a paraphyletic grade sister to a group of Peninsular Malaysian and Indonesian *Rafflesia* species. Monophyly of Bornean *Rafflesia* species is neither supported by resampling methods, nor was it recovered in all most parsimonious trees. However, given that overall branch support has increased with more data, and that the level of homoplasy is low, we anticipate the currently presented topology will be robust to further data inclusion. Furthermore, adding three Peninsular Malaysian and Philippine species did not distort the respective monophyly of these groups.

Our current age estimates of Rafflesiaceae divergences corroborate those previously obtained with less data and less precise calibration (Barkman et al., 2008: Figs. 1 and 2; Fig. 4, this study), and a Late Cretaceous origin and initial divergence of Rafflesiaceae from Euphorbiaceae-like ancestors seems likely. However, life history and between-lineage heterogeneity in rates of molecular divergence may impose biases in molecular divergence time estimation (Berbee and Taylor, 2010; Smith and Donoghue, 2008), and for this reason fossil calibration is of outmost importance. As no Rafflesiaceae fossils are known, we had to rely on secondary calibration, which could potentially be a source of error in our analyses. Moreover, holoparasitic plants have been shown to be divergent in many parts of the genome (e.g. Nickrent et al., 1998), and we anticipate that other more reliable calibration points and additional genetic markers will refine our initial estimates, in particular for the earliest diverging lineages of this enigmatic plant family. Likewise, inferred ancestral areas become increasingly imprecise the older the divergence. Therefore, pre-Miocene inferences made here should be regarded as preliminary.

4.2. Floral morphological evolution interpreted in a molecular phylogenetic context – A general lack of morphological synapomorphies

As predicted from their distinct morphologies (Fig. 1), the respective monophyly of the three genera, *Rafflesia*, *Rhizanthes* and *Sapria*, is strongly supported (Fig. 3). However, the sister relationship of *Rafflesia* and *Rhizanthes*, which has been shown in several previous molecular studies (e.g. Barkman et al., 2008; Nickrent et al., 2004; Wurdack and Davis, 2009), contrasts predictions from morphology that suggested *Rafflesia* and *Sapria* should be closely related (Beaman et al., 1992; see also Fig. 1, this study). *Rafflesia* and *Sapria* share perigone lobes that are similarly shaped, reddish colored, often white warty and imbricate in bud. In contrast, *Rhizanthes* has non-warty perigone lobes that are shaped differently from those of *Rafflesia* and *Sapria*, terminating in stiff appendages, different in color (often appearing white), and valvate in bud. In both *Rafflesia* and *Sapria*, a central diaphragm (an inverted bowl-like structure; Fig. 1a–d) encloses the central portion of the flower except for the aperture. *Rhizanthes* lacks the diaphragm and aperture (Fig. 1e–f). Anther dehiscence is via a single pore in *Rafflesia* and *Sapria* whereas *Rhizanthes* has two. Thus, the numerous shared similarities of *Rafflesia* and *Sapria* must be interpreted as symplesiomorphies, not synapomorphies, and the distinctive characteristics of *Rhizanthes* must be recently evolved apomorphic features given the context of the phylogenetic patterns confirmed here (Fig. 3).

The respective monophyly of the Bornean, Indonesian, Peninsular Malaysian and Philippine clades of *Rafflesia* (Fig. 3) is not reflected in the morphology of the species. Rather, there appears to be a strikingly high level of homoplasy. For example, the Java endemic, *Ra. rochussenii*, and the Borneo endemic, *Ra. tengku-adlinii*, are both small (c. 20 cm in diameter) with perigone and diaphragm orange-red throughout (no white warts) and processes few or lacking (see Fig. 1a and b for the position of these characters). Also, a recently described Philippine species, *Ra. aurantia* Barcelona, Co and Balete, was described with the above mentioned characteristics and was reported to be similar to *Ra. tengku-adlinii* (Barcelona et al., 2009a). Likewise, large conspicuous white warts (although differently shaped) on intermediate sized flowers is a feature shared by the non-monophyletic Sumatra endemic, *Ra. hasseltii*, the Malay Peninsula endemic, *Ra. cantleyi*, the Bornean endemic, *Ra. pricei*, and the Philippine endemic, *Ra. schadenbergiana* Göpp. Although not studied here, preliminary molecular phylogenetic results recently reported in Barcelona et al. (2009a) indicate that the Philippine *Ra. schadenbergiana* groups with the other Philippine rafflesias and to the exclusion of non-Philippine rafflesias. Homoplasy in floral size has already been discussed thoroughly in Barkman et al. (2008). Based on these relationships among species, homoplasy of floral morphology seems to be the rule more than the exception in *Rafflesia*.

Although morphologically distinct, *Sapria ram* resembles *S. poilanei* more than either resembles *S. himalayana* (Bänziger and Hansen, 1997). *Sapria ram* and *S. poilanei* are both small, about half the size of *S. himalayana* in most floral attributes, they both have white warts distributed basally on wine-red lobes, whereas warts of *S. himalayana* are yellow and are distributed evenly on blood-red lobes, and their disks are walled whereas the disk of *S. himalayana* is not. Thus, given the phylogenetic sister-relationship between *S. poilanei* and *S. himalayana* (Fig. 3), the shared similarities of *S. ram* and *S. poilanei* must be interpreted as symplesiomorphies.

Rhizanthes deceptor H. Bänziger and B. Hansen is strongly supported as sister to *Rh. infanticida* (Fig. 3). Yet, in overall floral morphology, *Rh. deceptor* and *Rh. lowii* are the most similar of the three. For example, *Rh. deceptor* and *Rh. lowii* have stiff hairs (bristles) on their tepals, whereas hairs present on the tepals of *Rh. infanticida* and *Rh. zippelii* (Blume) Spach (the latter not included in the present study) are tangled and fine (furry hairs) (see Fig. 1e–f for position of these hairs; Bänziger and Hansen, 2000). The latter two species are also generally smaller and have fewer anthers (Bänziger and Hansen, 2000). Given the phylogenetic relationships presented here (Fig. 3), stiff hairs, bigger flowers and more anthers represent, again, symplesiomorphic character states.

Thus, our molecular phylogeny of Rafflesiaceae (Fig. 3) depicts a general lack of morphological synapomorphies as well as a high level of morphological homoplasy in *Rafflesia*. These results call for a more detailed study to discern potential morphological synapomorphies. However, a robust evaluation of currently recognized morpho-species would also require multiple accessions covering most of the geographic distribution of every species and preferably, additional, more variable genetic markers. Future studies should also aim to include the recently rediscovered *Rafflesia schadenbergiana* as well as recently described Philippine and Indonesian species that could not be included here (e.g. Barcelona et al., 2009b; 2007; 2008; Madulid et al., 2005, 2006; Susatya et al., 2006).

The fact that similar floral sizes and morphologies have evolved independently, especially within *Rafflesia*, strongly suggests that there has been selection for similar traits in different geographic areas. Presumably, pollinators are the agents of selection and the independently evolving traits in distinct geographic regions reflect actions of either the same fly species or different species with similar preferences.

4.3. Historical biogeography

As expected, the older the node the larger the uncertainty in ancestral area inference (Fig. 4), and obviously, little can be concluded from our results about area of origin of Rafflesiaceae, or any of its three component genera, except that they all likely originated in Southeast Asia. Terrains corresponding to today's continental Southeast Asia and Sundaland have constituted a continuous area for most of the time since the Jurassic, about 150 MyBP (Metcalfe, 1998: p. 38), except for the Philippines, which became part of Southeast Asia much later (Hall, 1998). However, during the Cenozoic, physical barriers were at times present between Sundaland and the continent, and may have promoted the disjunction of *Sapria* in the seasonal climates of continental Southeast Asia and *Rafflesia* and *Rhizanthes* in the more consistently wet forests of Sundaland. For instance, a proto-South China Sea separated the Southwest Borneo terrane from Indochina during the Cretaceous/Tertiary-boundary (c. 65 MyBP; Metcalfe, 1998), and from the Paleocene to Late Oligocene there was apparently no continuous land connection between Sundaland and the continent (Hall, 1998: Fig. 6). By the Oligocene–Miocene boundary, land connection was reestablished through the Isthmus of Kra, but this low and narrow piece of land that connects the Malay Peninsula with mainland Southeast Asia (see Fig. 2a) was frequently inundated. Sundaland, however, constituted a continuous area from the Late Cretaceous to the beginning of the Pliocene (c. 5 MyBP) (Hall, 1998; Outlaw and Voelker, 2008), and there is no evidence for a vicariant explanation for the *Rafflesia–Rhizanthes* divergence. Nevertheless, a Late Cretaceous appearance of the rainforest-adapted Rafflesiaceae ancestor (assuming physiological uniformitarianism) in Southeast Asia supports the existence of closed-canopy tropical rainforests in this region long before the Cretaceous-Tertiary boundary (65.5 Mya), as was recently suggested by Davis et al. (2005) and supported by others (e.g. Smith et al., 2008).

The subsequent long period of no net diversification (c. 73–12 MyBP) followed by a punctuated increase in diversification rate in all three genera (Figs. 4 and 5) demands an explanation. Scenarios of old stem ages and recent crown ages either imply long periods of stasis or high levels of extinction among early diverging lineages, or a combination of both (Smith et al., 2008). A good fossil record can distinguish real stasis from extinction(s), but as no Rafflesiaceae fossils are known, other sources of information must be sought. The curve shape of a lineage through time (LTT) plot can be informative about the cause of such long branches. Simulation studies have shown that mass extinctions create antisigmoidal curves (see Crisp and Cook, 2009: Fig. 3), and that the inferred extinction event precedes the end of the plateau. Inspection of the LTT plot in Fig. 5a reveals a striking resemblance to the antisigmoidal curve presented among the theoretical LTT plots in Crisp and Cook (2009: Fig. 1F), i.e. it rises steeply at first, curves over to a plateau, then rises steeply again to the present. This suggests a major extinction event prior to the abrupt increase in diversification of the three genera at about 12 MyBP (Fig. 5). A dramatic reduction in rainforest cover in Southeast Asia between 35 and 20 MyBP (Morley, 2007) may have caused Rafflesiaceae to become strongly bottlenecked. Both prior to and after this dramatic reduction in rainforest cover were long periods of rainforest-favorable conditions (Morley, 2007). Thus, the abrupt post-bottleneck increase in diversification rate, most pronounced in *Rafflesia* (Fig. 5b), could be explained by Mid-Miocene to Pliocene rainforest-favorable conditions (Morley, 2007). Mass extinctions are commonly thought to stimulate an adaptive radiation manifested by a sharp increase in the rate of diversification (Benton and Emerson, 2007). Similar cases of recent radiations in ancient lineages have been reported from other groups of plants (e.g. García-Maroto et al., 2009; Smith et al., 2008; Su and Saunders, 2009), and a high level of extinction among earlier diverging lineages has been the preferred explanation for the long period of no-net diversification.

As opposed to the older nodes, uncertainty in age estimates of Miocene or younger nodes is very low (Fig. 4), and inference of biogeographic history can more safely be drawn. The crown group age of *Rafflesia* was estimated to about 12 MyBP, and the first intrageneric divergence included the branching off of the well-supported monophyletic Philippine *Rafflesia* lineage. Intra-area (within unit area) diversification originated already by the Late Miocene in the Philippines, much earlier than within the other unit areas, which were mostly of Pleistocene age (Fig. 4). The Philippines represent an island system with a history detached from that of the Sunda Shelf and attained its current configuration as late as the Early Pliocene (Outlaw and Voelker, 2008). *Rafflesia* probably reached the Philippines during the Mid-Miocene (Fig. 4) via the Sulu archipelago (Fig. 2a), a now almost extinct volcanic arc (Hall, 1998). Widespread volcanism in the eastern Philippines throughout the Miocene and Pliocene created many small islands which were interconnected for only short periods of time (Outlaw and Voelker, 2008), which may have promoted speciation in Philippine rafflesias. The lack of pre-Pliocene divergence among the Sundaland *Rafflesia* species (Fig. 4 and Table 2) supports the existence of a barrier-free continuous Sunda Shelf until the beginning of the Pliocene (c. 5 MyBP; Outlaw and Voelker, 2008). Pliocene

diversification of *Rafflesia* appear to be largely vicariant, as reflected by the reciprocal monophyly of the Bornean, Indonesian (Sumatra and Java) and Malay Peninsular *Rafflesia* species (Fig. 4). Given the estimated age of these inter-clade divergences, and clear geographic pattern, it is likely that Pliocene fragmentation of the Sunda block caused these inter-area divergences. It was a dramatic increase in sea-level that separated the previously continuous Sunda Shelf, culminating about 3 MyBP (Zhong et al., 2004). Furthermore, the Southeast Asian islands were smaller than at present due to late Pliocene uplift (Hall, 1998), which made the inter-island sea-barrier even greater. Also, other studies of Southeast Asian plants and animals suggest a deep history of vicariant evolution between the islands and mainland that probably predate the Pleistocene and correspond with Pliocene fragmentation of Sundaland (e.g. Barkman and Simpson, 2001; Cannon and Manos, 2003; den Tex et al., 2010; Gorog et al., 2004). It is noteworthy that several species of Southeast Asian rodents, the group of animals to which the most likely Rafflesiaceae seed-dispersers belong, show the same level of area-endemism, no signs of migrations across Pleistocene landbridges, and the same pattern of Pliocene vicariant history (Gorog et al., 2004).

Pleistocene sea-level fluctuations associated with glacial intervals have been considered the major factor in the development of biogeographic patterns (or the lack thereof) in Sundaland (Moss and Wilson, 1998). Periodic low sea-levels during the Pleistocene created landbridges between islands on the Sunda Shelf and continental Southeast Asia (Bird et al., 2005, and references therein), allowing for inter-area dispersals in many groups of plants and animals (e.g. Ridder Numan, 1996; Van Welzen, 1994). Gene flow between closely related taxa would be expected from such inter-area migration, which would erase pre-existing biogeographic patterns on the Sunda Shelf. No Pleistocene inter-area dispersals are evident from our results, as indicated by the respective monophyly of the Rafflesia species within each unit-area (Figs. 3 and 4). The most recent species divergences in Rafflesiaceae are concordant with Pleistocene oscillations in climate and sea-level, but they are all intra-area divergences (Fig. 4). Also, absence of *Rafflesia* on Palawan, despite land-connections to Borneo at several times during the Pleistocene (Outlaw and Voelker, 2008), further supports that inter-area dispersals of Rafflesiaceae were pre-Pleistocene. Different views exist with regard to type of vegetation cover of the Pleistocene landbridges – forest or savannah (reviewed in Bird et al., 2005). A savannah corridor would be a dispersal route for many organisms, but it would also serve as a barrier to the dispersal of rainforest-dependent species. Because Rafflesiaceae are assumed to be confined to rainforest vegetation, the lack of Pleistocene inter-area migration is supportive of dry savannah rather than rainforest cover of the Pleistocene landbridges, as concluded by Bird et al. (2005). Outlaw and Voelker (2008) described habitat shifts associated with sea-level fluctuations in Southeast Asia as follows: Rainforests retracted during periods of low sea-levels (cold and dry) and expanded during periods of high sea-levels (warm and humid). Thus, at all times during the Pleistocene, there were barriers to inter-island dispersal of rainforest taxa, either as dry savannah vegetation or sea (inundated landbridges).

Co-evolution between host and parasite may also have promoted diversification in Rafflesiaceae. The strong phylogeographic pattern in our results (Fig. 4), however, indicates that inter-specific diversification in *Rafflesia* is more attributed to geography than to host – i.e. that parasite speciation did probably not involve concurrent host speciation.

4.4. Character displacement

The independent evolution of large floral sizes and divergent floral ornamentation patterns seen in partially sympatric *Rafflesia* species could be due to selection for character displacement. If effective, this character displacement would potentially provide barriers to inter-specific pollen transfer, and could explain why natural hybrids have never been verified even though there is little flowering phenological differentiation among species (Nais, 2001). Character displacement was the preferred model describing floral size evolution within *Rafflesia* as described above. Furthermore, the data we present on floral size variation in *Ra. cantleyi* and *Ra. kerrii* cannot reject such a hypothesis. If anther and stigma chamber sizes generally scale allometrically with floral size, as it appears they do in *R. cantleyi* and *R. kerrii*, then the variation observed among sympatric *Rafflesia* could be explained as a result of selection to avoid inter-specific hybridization. Along with floral size, other characteristics do vary such that, in sympatric situations, the small flowered species have white warts while the large diameter species do not. While this hypothesis of character displacement can explain the floral evolution pattern within *Rafflesia*, it does not necessarily indicate that speciation events were driven by this process.

Increased organ size is often associated with increase in ploidy level (e.g. Ramsey and Schemske, 2002). However, differences in ploidy level are unlikely to account for the rapid divergences of small and large flowered species from intermediate-sized ancestors in *Rafflesia*, because chromosome counts made on the largest species, *Ra. arnoldii,* show that it has the same number of chromosomes as the smaller flowered species, *Ra. patma* Blume (both $n = 12$; Olah, 1960), and the sister genus *Rhizanthes* ($n = 11$; Meijer, 1997). This does not preclude a potential role of localized genomic variation in DNA content though, which has also been implicated in floral size evolution (Meagher et al., 2005). Plant and organ size may also be correlated with altitude, with larger individuals/organs occurring at lower elevation and smaller individuals/organs found at higher elevations. However, this is not the case for *Rafflesia* species because there is considerable overlap in their altitudinal distributions (Nais, 2001), and analysis of the relationship of floral size and highest recorded elevation for each species resulted in an r of only -0.12 ($P = 0.7$). It is possible that, instead of character displacement to avoid gamete wastage, pollinator species have selected for divergent floral sizes corresponding to their differing preferences for carcass sizes, color, and stage of decomposition as has been reported for some calliphorid and sarcophagid flies (Braack, 1987; Kneidel, 1984). Thus, it may be that large carrion-fly pollinated plants are in a co-evolutionary race with their deceived pollinators resulting in the evolution of different floral/inflorescence sizes (Davis et al., 2008).

5. Conclusion

The present study elucidates the scenario in which the rainforest-adapted holoparasitic Rafflesiaceae evolved and radiated. A general lack of morphological synapomorphies and a high level of morphological homoplasy are indicative of similar patterns of pollinator-based selection in different geographic areas and are consistent with a model of character displacement. A Late Eocene to Miocene dramatic reduction in rainforest cover probably imposed a severe bottleneck on Rafflesiaceae and thus may have caused the c. 60 million years of no apparent net diversification. The abrupt post-bottleneck increase in diversification rate is most pronounced in *Rafflesia* and may be explained by Mid-Miocene to Pliocene rainforest-favorable conditions as well as natural selection promoting character displacement for *Rafflesia* flower size. Late Miocene to Early Pliocene rise in sea-level probably caused the vicariant inter-area diversification evident from the geographically structured *Rafflesia* phylogeny. Lack of inter-area migrations during Pleistocene periods of low sea-level suggests savannah rather than rainforest covered landbridges at this time.

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