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A phylogenetic and biogeographic study of *Rafflesia* (Rafflesiaceae) in the Philippines: Limited dispersal and high island endemism

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ABSTRACT

Rafflesia (Rafflesiaceae) is a small endo-holoparasitic Asian plant genus known for its exceptionally large flowers, rare species, and high island endemism. In this study, phylogenetic (parsimony and Bayesian inference) and biogeographic (BioGeoBEARS) analyses of DNA sequence data (*atp*6 and *mat*R genes, and *nad*1 B-C intron from the mitochondrial genome, and the nuclear ribosomal internal transcribed spacer) were used to reconstruct the phylogenetic relationships among 12 of the 13 known Philippine *Rafflesia* species and to determine the timing and pathways of their diversification. The results of these analyses confirm those of previous *Rafflesia* studies (which were largely focused on non-Philippine species) in finding pronounced biogeographic patterns. They suggest that dispersal between islands has been relatively uncommon, and indicate that the high island endemism of *Rafflesia* is a result of poor inter-island dispersal abilities. The results further suggest that its ancestral range might have been in Borneo, and that its lineages and species evolved earlier and more gradually than previously assumed.

1. Introduction

Rafflesia R.Br. is the most species-rich of the three genera in the tropical Southeast Asian plant family Rafflesiaceae (Malpighiales; 37–44 species; Nickrent, 1997 onwards; Bendiksby et al., 2010). Its species are known for their exceptionally large flowers and obligate endo-holoparasitic relationship with their host plants, which are exclusively vines of the genus *Tetrastigma* (Miq.) Planch. (Vitaceae; Pelser et al., 2016). Between 30 and 37 *Rafflesia* species are currently recognized (Nickrent, 1997 onwards), many of which are rare and threatened by habitat destruction, degradation, and fragmentation (Meijer, 1997; Hidayati et al., 2000; Nais, 2001; Barcelona et al., 2009a; Mursidawati et al., 2015; Wicaksono et al., 2016). Their flower size, rarity, as well as parasitic lifestyle and associated highly specialized morphology make this genus valuable for studying various aspects of the biology and evolution of parasitism (e.g., Nikolov et al., 2013; Xi et al., 2012a, 2013; Molina et al., 2014; Barkman et al., 2017; Nikolov and Davis, 2017; Twyford, 2017; Ng et al., 2018; Wee et al., 2018). The research presented here aims to further develop previously published phylogenetic and biogeographic hypotheses relating to the diversification of *Rafflesia* (Barkman et al., 2008; Bendiksby et al., 2010) and to use these as a framework for improving our understanding of the evolution of these most specialized parasites.

Rafflesia has high island endemism: all but six species (*R. arnoldi* R.Br., *R. cantleyi* Solms, *R. gadutensis* Meijer, *R. patma* Blume, *R. rochussenii* Teijsm. & Binn., *R. speciosa* Barcelona & Fernando) are endemic to individual islands in the Malesian archipelago or to the Malay Peninsula (Nickrent, 1997 onwards; Nais, 2001; Hidayati and Walck, 2016; Pelser et al., 2017). Because *Rafflesia* plants and their *Tetrastigma* hosts grow in various tropical rainforest ecosystems (Nais, 2001; Barcelona et al., 2009a, 2011), and the hosts appear to be relatively common and widespread (Nais, 2001; Pelser et al., 2016), it is perhaps unlikely that the high island endemism is a result of very narrow environmental tolerances, or host species with small distribution ranges (Pelser et al., 2018). Instead, it is more likely that *Rafflesia* seeds are poorly dispersed between islands (Pelser et al., 2017, 2018). Although various animals have been proposed as seed dispersers of *Rafflesia* (Teijsmann, 1856; Justesen, 1922; Kuijt, 1969; Emmons et al., 1991; Bouman and Meijer, 1994; Nais, 2001; Bänziger, 2004), our field observations suggest that myrmecochory (dispersal by ants) might be the primary means of seed dispersal (Pelser et al., 2013, 2018), and it is therefore possible that seed dispersal across water is rare, reducing the

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chances that seeds are carried across the seas and straits that separate islands. This hypothesis finds support in the results of a population genetic study of *R. speciosa*, the only Philippine species that is found on two different islands. Pelser et al. (2018) showed that *R. speciosa* populations on Panay and Negros Islands are genetically well differentiated, suggesting limited recent gene flow across the narrow sea strait that separates the two islands.

If *Rafflesia* seeds are indeed primarily dispersed by ants, this would also explain data that suggest that *Rafflesia* species are also poor dispersers within islands, because myrmecochory is associated with very short seed dispersal distances compared to many other mechanisms (Gómez and Espadaler, 1998; Vittoz and Engler, 2007; Lengyel et al., 2009). For example, Barkman et al. (2017) showed that *R. cantleyi* and *R. tuan-mudae* Becc. populations have a pronounced genetic substructure: *Rafflesia* individuals sharing the same host plant are genetically differentiated from those growing on different but nearby hosts. That study, as well as population genetic data from *R. lagascae* Blanco and *R. speciosa* (Pelser et al., 2017, 2018), also suggest poor genetic connectivity among *Rafflesia* populations located more than ~20 km from each other (Pelser et al., 2018). The restricted distribution of many *Rafflesia* species within islands also indicates that *Rafflesia* might be dispersal-limited. Whereas a few species are relatively widespread within an island (e.g., *R. lagascae*), most are known from one or only a handful of populations that are confined to small parts of their putative potential distribution range within an island (e.g., *R. aurantia* Barcelona, Co & Balete, *R. manillana* Teschem., *R. micropylora* Meijer, *R. schadenbergiana* Göppert ex Hieron., *R. tengku-adlinii* Mat-Salleh & Latiff; Nais, 2001; Barcelona et al., 2009a; Pelser et al., 2017).

In their molecular phylogenetic study of Rafflesiaceae, Bendiksby et al. (2010) provide a biogeographic hypothesis for *Rafflesia*'s present-day distribution. They found that the 18 *Rafflesia* species included in their study form four geographically distinct clades (i.e. Borneo, Malay Peninsula, Philippines, Sumatra and Java), a deep pattern of relationships that is compatible with their apparent infrequent dispersal between islands and over larger distances. Bendiksby et al. (2010) suggest that *Rafflesia* was able to spread throughout Sundaland when it formed a continuous area prior to the Pliocene (c. 5.3 million years ago, Ma) at times when sea-levels were lower than at present. From Sundaland, it potentially found its way via the Sulu archipelago into the Philippines during the mid-Miocene (Bendiksby et al., 2010). During the late Pliocene, sea-level rises might have resulted in vicariance and subsequent speciation resulting in the four geographical clades (Bendiksby et al., 2010). Although land bridges among these areas potentially existed during colder periods with low sea-levels in the Pleistocene, these corridors might have been vegetated with savannah instead of tropical rain forest, and this might have prevented rain forest-adapted *Rafflesia* from dispersing between islands (Bendiksby et al., 2010).

The pronounced pattern of island endemism of *Rafflesia* is particularly obvious in the Philippines, where 12 of the 13 presently known species are endemic to single islands (Pelser et al., 2011 onwards, 2017, 2018). The Philippine species are found on five of the major islands of the archipelago: Luzon, Mindanao, Negros, Panay, and Samar (Fig. 1). *Rafflesia* has not been recorded on any of the other Philippine islands, including the relatively large islands of Bohol, Cebu, Mindoro, and Palawan. Using DNA sequences of 12 of the 13 Philippine *Rafflesia* species, and previously generated sequence data for Sundaic (i.e. non-Philippine) species, we aimed to reconstruct the phylogenetic relationships among Philippine *Rafflesia* and use these data to explore the biogeographic history of the genus in the Philippines. The ultimate goal of our studies was to contribute to discussions about the high island endemism of *Rafflesia* and the narrow distribution ranges of most species of this genus (e.g., Barcelona et al., 2009a; Bendiksby et al., 2010; Barkman et al., 2017; Pelser et al., 2017, 2018) by determining the number of inferred inter-island dispersal events within the Philippines. Finding a low number of such dispersals would be compatible with our hypothesis that *Rafflesia* disperses poorly between islands.

2. Material and methods

2.1. Specimen sampling

Tissue samples from flower buds or opened flowers were collected in silica gel for 12 of the 13 currently recognized Philippine *Rafflesia* species (Pelser et al., 2011 onwards). We were not able to obtain tissue of *R. aurantia*, which is known from a single remote population in Quirino Province in Luzon (Barcelona et al., 2009b; Fig. 1). Except for *R. mira* and *R. schadenbergiana*, more than one sample per species was included in our analyses. The details of our sampling strategy are described by Pelser et al. (2017). Voucher specimens were deposited at CAHUP, CANU, PNH, and SIU (Table S1).

2.2. DNA sequencing and alignment

DNA was extracted from tissue samples using the DNeasy plant mini kit (Qiagen, Germantown, Maryland, USA). Four DNA regions were selected for sequencing: the *atp*6 and *mat*R genes and *nad*1 B-C intron of the mitochondrial genome, and the nuclear ribosomal Internal Transcribed Spacer (ITS-1, 5.8S, ITS-2) region (hereafter referred to as ITS). These regions were chosen to enable us to combine our DNA sequence data with the data set generated by Bendiksby et al. (2010). Because the *nad*1 B-C region was acquired by Rafflesiaceae via horizontal transfer from Vitaceae (Davis and Wurdack, 2004), this region was only used to resolve relationships among Rafflesiaceae taxa and not between Rafflesiaceae and members of other families (see below). Its use for resolving the Rafflesiaceae phylogeny is appropriate, because the horizontal gene transfer event took place before the three Rafflesiaceae genera diverged (Barkman et al., 2008). Bendiksby et al. (2010) also included in their analysis sequences of a putative plastid ribosomal 16S gene. These fragments were less than 400 bp long and highly divergent compared to photosynthetic angiosperms, thus we presume they are products of horizontal gene transfer, likely residing in the nucleus. Because it is presently unknown how often and when in the evolutionary history of Rafflesiaceae this event took place, we excluded these data from our study to avoid the risk of obtaining erroneous phylogenetic patterns resulting from incorrect assumptions regarding homology.

The *atp*6 region was PCR-amplified and sequenced with primers atp6F and atp6R (Barkman et al., 2008). For the *mat*R region, we used the matR 5′ (Anderberg et al., 2002), matR forward 1, and matR forward 2 (Barkman et al., 2004) forward primers, and the matR 3′ (Anderberg et al., 2002), matR reverse 2 (Barkman et al., 2004), and the newly developed matR reverse 3 (5′-CAAGCCCTCGAGCCTCC TTT-3′) reverse primers. The *nad*1 B-C region was amplified using the nad1 12F1 (Demesure et al., 1995) and nad1 Raf F3 (Barkman et al., 2008) forward primers and the nad1 12R1 (Demesure et al., 1995), nad1 Raff R2, and nad1 Raff R3 (Barkman et al., 2008) reverse primers. We used forward primers ITS-I (Urbatsch et al., 2000) and ITSA (Blattner, 1999), and reverse primers ITS4 (White et al., 1990) and ITSB (Blattner, 1999) for the ITS region. Most PCR amplifications were performed in a 15 μL volume reaction with 0.6 μL of each forward and reverse primer (10 mM), 1.5 μL of HotMaster (5PRIME) Taq buffer with 1.5 μL of 10X magnesium, 0.5 μL of combined dNTPs, 1.5 μL of 10x BSA, and 0.1 μL of HotMaster Taq DNA polymerase. For a few samples, a slightly modified protocol was used with either KappaTaq ReadyMix DNA Polymerase (Kappa Biosystems, Wilmington, Massachusetts, USA) or GoTaq Flexi DNA Polymerase (Promega, Madison, Wisconsin, USA). Amplification was performed using the following conditions: 5 min at 94 °C; 35 cycles of 94 °C for 30 s, 1 min at 50 °C (30 s at 52 °C for ITS), 70 °C for 1 min (additional 1 min/1kb); followed by a final extension at 70 °C for 5 min.

PCR products were purified with either ExoSAP-IT (GE Healthcare) or the Wizard SV Gel and PCR Clean-Up System (Promega). Cycle sequencing was carried out with BigDye Terminator v3.1 (Applied

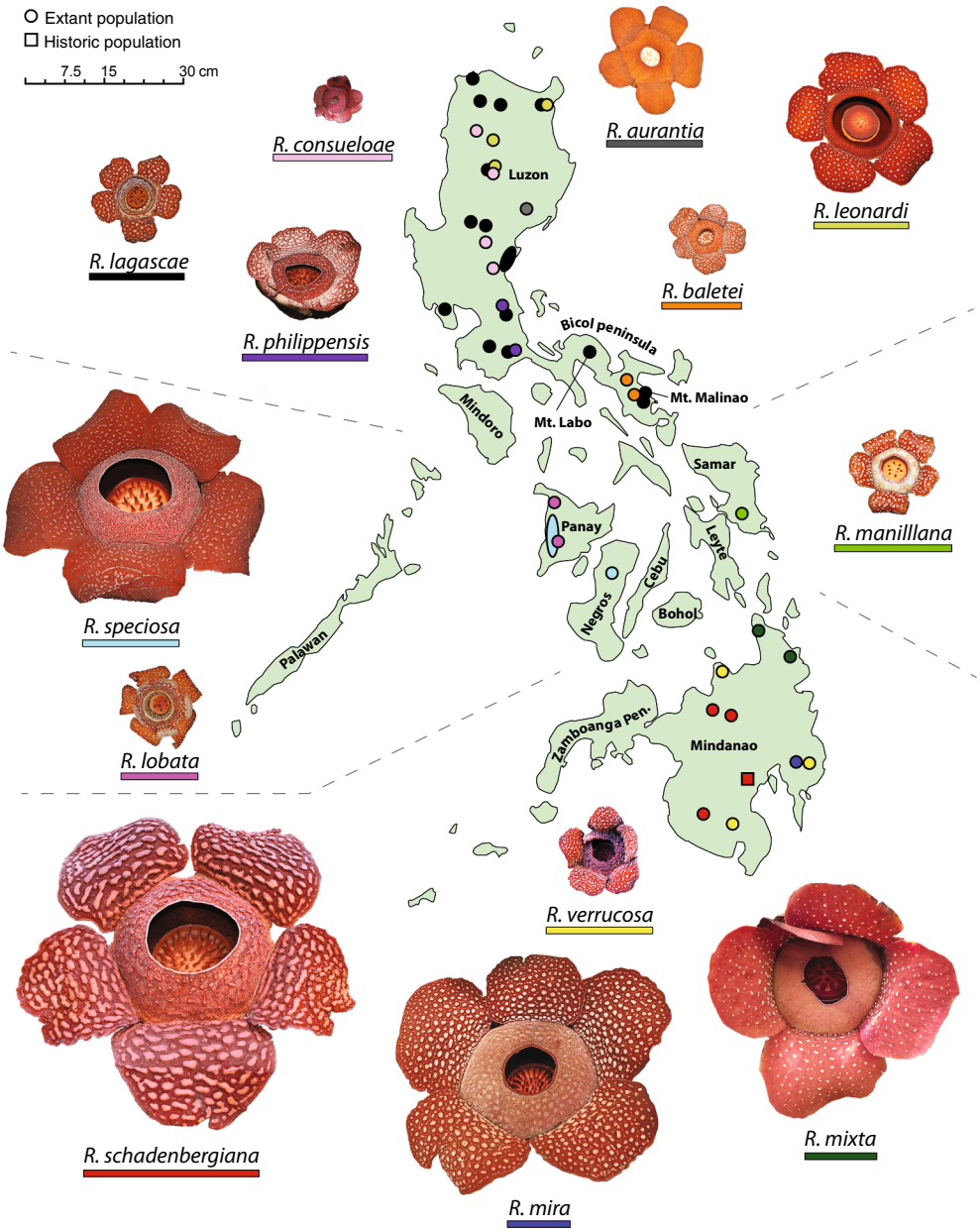

Fig. 1. Distribution map of Philippine *Rafflesia*. Flowers shown at relative sizes, with scale bar. Dotted lines indicate groups of *Rafflesia* species growing in Luzon, Mindanao, Panay & Negros, and Samar.

Biosystems, Foster City, California, USA) using the same primers as used for PCR amplification. For some samples, BDX64 Enhancing Buffer (MCLAB, San Francisco, California USA) was used. Amplification was performed with the following cycles: 1 min (3 min for BDX64) at 96 °C; 25 cycles (30 cycles for BDX64) of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 75 s (2 min for BDX64). Amplification products were cleaned with Agencourt CleanSEQ (Beckman Coulter, Beverly, Massachusetts, USA) or an ethanol/EDTA/sodium acetate precipitation following the BigDye Terminator v3.1 manual. The sequenced samples were run on an ABI 3130 or ABI 3130xL Genetic Analyzer. Sequencher v.4.8 (Gene Codes, Ann Arbor, Michigan, USA) or Geneious v.6.1.7 (Biomatters, Auckland, New Zealand) was used for trace file editing. Edited sequences are deposited in GenBank (Table S1).

For each of the four DNA regions, the newly generated sequence data were aligned in Geneious v.6.1.7 (MUSCLE Alignment option; Edgar, 2004) with the alignments used by Bendiksby et al. (2010). These combined alignments (Supplementary data 1) were subsequently manually edited to remove inconsistently aligned motifs (Kelchner, 2000). The newly sequenced specimens of some Philippine *Rafflesia* species did not show genetic differences for any of the four DNA regions among multiple accessions of the same species. For these species, only one specimen was included in the final version of our alignments. The three accessions of *Sapria himalayana* Griff. in the Bendiksby et al. (2010) data set were replaced with a single consensus sequence in which nucleotides at polymorphic positions were replaced with ambiguous bases (Pelser et al., 2007), an approach we also used for the two accessions of *Rhizanthes infanticida* Bänziger & B. Hansen. After confirming with preliminary Bayesian Inference (BI) analyses (see below for methodology) that the four accessions of Philippine *Rafflesia* species that were sequenced by Bendiksby et al. (2010) (i.e. *R. lobata* R. Galang & Madulid, *R. manillana* (now considered a specimen of *R. lagascae*), *R.* sp. (now identified as *R. baletei* Barcelona & Cajano), and *R. speciosa*) each formed a clade with the newly sequenced specimens of these species, we removed those accessions from the alignments.

There is no fossil record for Rafflesiaceae, and molecular dating attempts that could provide the approximate timing of key events in the

biogeographical history of Philippine *Rafflesia* therefore require external calibration points. For this purpose, we added *mat*R sequences and, when available, *atp*6 and 5.8S sequences of 24 Malpighiales taxa and a *Euonymus* L. (Celastrales) species as an ultimate outgroup to our alignments (Table S1). These taxa were selected using the calibration points and results of Xi et al. (2012b) and Magallón et al. (2015). Sequences of the *nad*1 B-C region for these taxa were not used in our study because of their aforementioned horizontal transfer to an ancestral Rafflesiaceae species (Davis and Wurdack, 2004). ITS1 and ITS2 data from the non-Rafflesiaceae taxa could not be aligned with those of Rafflesiaceae, hence only sequences of the 5.8S region of the ITS cistron were used in our molecular dating analyses.

2.3. Phylogenetic analyses

The Gapcode.py v.2.1 Python script (distributed by Richard Ree, Field Museum, Chicago, Illinois, USA) was used to code indels as binary characters using the simple indel coding method of Simmons and Ochoterena (2000). BI analyses (methodology outlined below) of the separate *atp*6, *mat*R, *nad*1 B-C, and ITS alignments did not reveal phylogenetic incongruence among them that is well-supported (i.e. > 0.95 posterior probability). In addition, the individual BI consensus trees that were obtained from them were relatively poorly resolved and supported (Figs. S1-4). Therefore, the alignments of the four regions were concatenated into a single data set which was used for all subsequent phylogenetic analyses. This was done with the aim of using all available data for resolving relationships among *Rafflesia* species. Genetic diversity data for the separate and concatenated alignments can be found in Table S2.

MP analyses were carried out in TNT v.1.1 (Goloboff et al., 2008) using the Driven Search option with the default settings for Sectorial Searches (RSS, CSS, XSS), Ratchet, Tree Drifting and Tree Fusing; using 100 initial random addition sequences, and terminating the search after minimum length trees were found ten times. Bootstrap support was calculated with Poisson independent reweighting using 1000 replicates. BI analyses were performed using MrBayes v.3.2.5 (Ronquist et al., 2012), either on a laptop computer or via the CIPRES Science Gateway web portal (Miller et al., 2010). Following nucleotide substitution model selection using the Akaike information criterion in jModelTest v.2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012), the GTR + G model was chosen for the BI analyses. The Markov k model (Lewis, 2001) was used for indel characters. These analyses were performed using two independent, simultaneous runs. The Markov Chain Monte Carlo (MCMC) analyses (Geyer, 1991) were run with four chains per analysis, temperature settings of 0.01, and one tree saved every 100 generations. BI analyses were run until the average deviation of split frequencies between both simultaneous analyses reached a value below 0.01, suggesting potential convergence. The burn-in values were determined empirically from the likelihood values and the corresponding burn-in fractions were subsequently removed. Trees were visualized using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Bayes factor comparison (Kass and Raftery, 1995; Suchard et al., 2001; Brown and Lemmon, 2007) was used to test the hypothesis that the four *Rafflesia* species from Mindanao (*R. mira*, *R. mixta* Barcelona, Manting, Arbolonio, R.B. Caball. & Pelser, *R. schadenbergiana*, *R. verrucosa* Balete, Pelser, Nickrent & Barcelona) form a clade. We tested this hypothesis, because the results of our MP analyses place these four species in a poorly-supported clade (Fig. S5), which was not recovered by the BI analyses (Fig. 2). The latter instead resulted in a polytomy between *R. mixta*, the *R. mira-R. schadenbergiana-R. verrucosa* clade, and a clade formed by *R. lagascae*, *R. leonardi* and *R. manillana*. The Bayes factor is the ratio between the marginal likelihoods of two models. This statistic is commonly used in Bayesian phylogenetics to compare different evolutionary hypotheses (e.g., Xie et al., 2011; Valcárcel et al., 2014; Pinto et al., 2019; Sousa-Santos et al., 2019; Sánchez-Chávez et al., 2019). A value of $2\ln BF_{12}$ (twice the difference between the marginal likelihoods of the competing hypotheses) > 10 is considered as very strong support for hypothesis 1 and a value < −10 as strong support for hypothesis 2 (Kass and Raftery, 1995). To test the hypothesis that the four *Rafflesia* species from Mindanao form a clade, the marginal likelihoods resulting from two analyses, one with positive and one with negative constraints, were compared. The positive constraint analysis sampled only trees in which the four species from Mindanao form a clade. The negative constraint analysis sampled only those in which they do not form a monophyletic group. The means of the marginal likelihoods were estimated using a stepping-stone approach (Xie et al., 2011) in MrBayes. The stepping-stone sampling analyses were executed with two independent simultaneous runs of 50 steps with 200,000 generations within each step (a total of 10 million generations) and the posterior distributions were sampled once every 1000 generations. Ten thousand samples were obtained and these fell into 50 bins, one of which was the burn-in and was discarded. Convergence among independent runs of each step of the stepping-stone sampling was checked by examining the estimated marginal log likelihood values of the runs (Ronquist et al., 2011).

2.4. Divergence time estimations

The stepping-stone sampling method in MrBayes (same settings as above) was also used to test the hypothesis that the evolutionary rate of the taxa in our data set is constant (i.e. conforms to a strict clock-like model). Because the results of this analysis very strongly rejected the strict clock hypothesis (mean marginal likelihood of −15030.08 for the strict clock hypothesis vs. −15012.58 for the unconstrained hypothesis; $2\ln BF_{12} = 35$), divergence time estimations in BEAST v.2.5.1 (Drummond and Rambaut, 2007; Suchard and Rambaut, 2009) were performed using a relaxed clock model. Input files for BEAST (same data set as used for the MrBayes analyses but without gap-coded characters) were compiled with BEAUti v.2.5.1. We selected the GTR + G model of nucleotide substitution and a relaxed lognormal clock and chose Yule or Birth-Death models for the tree prior. We constrained the topology of the Rafflesiaceae clade to reflect the results of the BI analysis in MrBayes. However, following the results of a stepping-stone analysis (see above and reported in Results) the *Rafflesia* species from Mindanao were constrained to form a clade. There are no internal calibration points for Rafflesiaceae that can be used to inform divergence time estimations for this family and its lineages (Bendiksby et al., 2010). We therefore used external calibration points from a wide range of other Malpighiales families. Instead of performing novel phylogenetic analyses with these 'outgroups', we constrained the relationships among them and Rafflesiaceae following the results of previous studies that included a taxon and character sampling strategy that is much more appropriate for determining family-level relationships than our species-level phylogenetic data set. We constrained Rafflesiaceae to be sister to Euphorbiaceae + Peraceae (Davis et al., 2007; Wurdack and Davis, 2009; Xi et al., 2012b; Chen et al., 2016), and further constrained the relationships among the Malpighiales taxa following the topology of the Malpighiales phylogeny in Xi et al. (2012b). The calibration settings are reported in Table S3. The MCMC analyses were run for 90 million generations on the CIPRES Science Gateway. Parameter values were recorded every 1000 generations. Tracer v.1.6.0 was used to evaluate convergence. The first 10% of generations were discarded as burn-in. All analyses resulted in effective sampling sizes (ESS) > 200 for all parameters. TreeAnnotator v.2.5.1 was used to calculate maximum clade credibility trees, the mean node ages and their 95% highest posterior density intervals (HPD). These were visualized using FigTree.

2.5. Biogeographical analyses

The maximum clade credibility tree obtained using the Yule model in BEAST was used as the dated input tree for a biogeographical analysis with the R (R Core Team, 2018) package BioGeoBEARS v.1.1 (Matzke, 2013). This tree was pruned to exclude all non-Rafflesiaceae OTUs and to include only a single OTU for each Rafflesiaceae species.

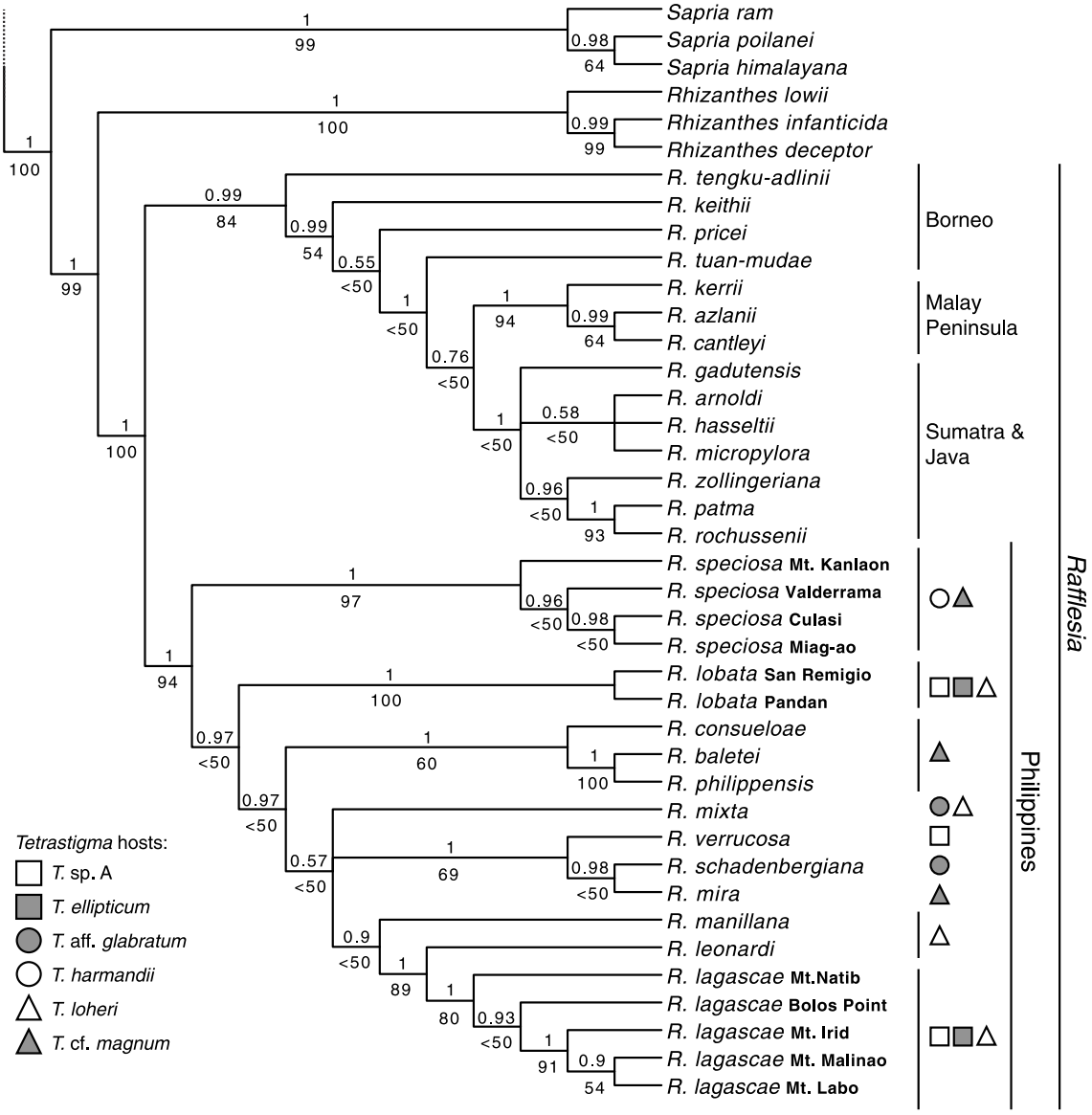

Fig. 2. Bayesian Inference phylogeny of the combined *atp*6, *mat*R, *nad*1 B-C and ITS data set. Outgroups not shown. Posterior probabilities above the branches, Maximum Parsimony bootstrap percentages below the branches. *Tetrastigma* host species shown for Philippine *Rafflesia* as per Pelser et al. (2016) and this study (for *R. consueloae*).

However, two OTUs of *R. lagascae* were included: one representing *R. lagascae* sensu stricto and one representing the Mt. Labo population of *R. lagascae* (Fig. 1). This was done because the results of Pelser et al. (2017) indicated that the Mt. Labo population might constitute a distinct, cryptic species that is genetically distinct from the other populations currently classified as *R. lagascae*. Using information about the current distribution ranges of Rafflesiaceae species, 10 biogeographically meaningful areas were defined for the ancestral range analyses: (1) mainland SE Asia, (2) Malay Peninsula (i.e. peninsular Thailand and Malaysia), (3) Sumatra and Java, (4) Borneo, (5) mainland Luzon, (6) the Bicol peninsula of Luzon, (7) Samar, (8) Panay, (9) Negros, and (10) Mindanao (5–10 are areas within the Philippines). The maximum number of areas in the distribution range of each species was set at four. We considered this to be a sufficiently high number, because the present distribution ranges of Rafflesiaceae species do not contain more than two of the above areas. The AIC was used to select the model that best fits our data among the six available BioGeoBEARS models: DEC, DEC + J, DIVALIKE, DIVALIKE + J, BAYAREALIKE, BAYAREALIKE + J (Ronquist, 1997; Ree and Smith, 2008; Landis et al., 2013; Matzke, 2014). DEC is the likelihood-based Dispersal-Extinction Cladogenesis model implemented in the LAGRANGE software package (Ree and Smith, 2008). DIVALIKE is a likelihood version of the parsimony-based Dispersal-Vicariance Analysis model (Ronquist, 1997). BAYAREALIKE is a likelihood version of the Bayesian BayArea model (Landis et al., 2013). The '+ J' versions of these models include a founder-effect speciation parameter and this allows a descendant to occupy a different area than its immediate ancestor (Matzke, 2013). Likelihood Ratio Tests were used to compare the three pairs of nested models (e.g., DEC vs. DEC + J). Biogeographical Stochastic Mapping (BSM; Dupin et al., 2017) is a simulation approach that estimates the probability of ancestral biogeographical character states for each node of a phylogeny. An analysis with 100 BSMs and using the best-fit model was carried out to estimate the relative numbers of different biogeographical events.

3. Results

3.1. Phylogenetic analyses

MP (Fig. S5) and BI phylogeny (Figs. 2 and S6) estimation resulted in very similar hypotheses of the phylogenetic relationships among

Rafflesiaceae species, differing only in the resolution of clades that are poorly supported in both the MP bootstrap and BI consensus trees. *Rafflesia* is composed of two well-supported sister clades, one formed by all Philippine species (1.00 posterior probability (PP), 94% bootstrap support (BS)), and one composed of all Sundaic species (0.99 PP, 84% BS) included in our analyses. Within the Philippine clade, the two species from western Visayas (i.e. Panay and Negros; *R. lobata*, *R. speciosa*; Fig. 1) form a grade in which a clade composed of the species from elsewhere in the Philippines (0.97 PP, < 50% BS) is nested. The latter clade contains a relatively well-supported subclade (1.00 PP, 60 BS) composed of three *Rafflesia* species from mainland Luzon and the Bicol peninsula of Luzon: *R. baletei*, *R. consueloae* Galindon, Ong & Fernando, and *R. philippensis* Blanco. These are among the smallest of Philippine *Rafflesia* and have flowers with apertures that are relatively small compared to those of the other two species from Luzon (*R. lagascae*, *R. leonardi* Barcelona & Pelser) and a species from Samar (*R. manillana*), which also form a clade (0.90 PP, < 50% BS). The latter clade also contains a representative from the Mt. Labo population of *R. lagascae*, which forms a well-supported subclade with representatives of this species from other populations (1.00 PP, 89% BS). The four remaining species of Philippine *Rafflesia* are all from Mindanao. Three of these (*R. mira*, *R. schadenbergiana*, *R. verrucosa*) form a relatively well-supported clade (1.00 PP, 69% BS). In the results of the MP analyses (Fig. S5), the fourth Mindanao *Rafflesia* (*R. mixta*) forms a clade with these three species (< 50% BS), but this clade is not resolved in the BI consensus tree (Fig. 2). However, the results of a Bayesian stepping-stone analysis strongly favour the hypothesis that the four *Rafflesia* species from Mindanao form a clade (mean marginal likelihood −22165.81) over the non-monophyly of this species group (mean marginal likelihood −22172.95; $2\ln BF_{12} = 14.28$).

3.2. Divergence time estimations

The results of divergence time estimations using a relaxed-clock and a Yule model in BEAST are presented in Fig. 3. Similar results were obtained when a Birth-Death model was used (Table 1). The 95% Highest Posterior Density (HPD) ranges of the diversification events within Rafflesiaceae are relatively large. They suggest a Cretaceous origin of the family (95.5–106.6 Ma) and that *Rafflesia* diverged from *Rhizanthes* Dumort. between 52.1 and 83.8 Ma (mean 67.9 Ma). The speciation event that resulted in the Philippine and Sundaic *Rafflesia* clades might have taken place between 32.3 and 66.8 Ma (mean 49.8 Ma). The crown age of Philippine *Rafflesia* was resolved at 23.3–54.8 Ma (mean 39.0 Ma). Most extant species of Philippine *Rafflesia* have a mean stem age of between 9.1 and 20.9 Ma, placing their time of origin around the Miocene. *Rafflesia lobata* and *R. speciosa*, however, are potentially older (Eocene to early Miocene) and *R. baletei* and *R. philippensis* might be the youngest species of Philippine *Rafflesia* (up to 11.1 Ma, mean 3.9 Ma). In contrast, most of the Sundaic *Rafflesia* species (10 of 14) included in our analyses seem to be younger and have evolved in the Pliocene or more recently (mean ages 1.0–3.9 Ma).

3.3. Biogeographical analyses

The AIC identified DEC + J as the best fit model for our Rafflesiaceae data set (Table 2). A Likelihood Ratio Test showed that the addition of the jump dispersal parameter J to the DEC model results in a significantly higher log likelihood value ($p < 0.001$; Table S4). Biogeographical Stochastic Modeling using the DEC + J model suggests that most biogeographical events involve within-area speciation (61.4%; Table 3). Jump dispersal to a new area (i.e. founder-event speciation) accounts for 19.4% of all biogeographical events and range expansions are less common (11.9%). Only 7.2% of events are explained by vicariance (Table 3).

The results of the BioGeoBEARS analysis using the DEC + J model (Fig. 4) are not conclusive about the ancestral range of Rafflesiaceae; however, they indicate that the highest probability is that *Rafflesia* originated in Borneo. From there, it most probably dispersed once into the Malay Peninsula and then into Sumatra and Java, or vice versa. The presence of *R. arnoldi* in Sumatra and Borneo is explained by a range expansion event from the former to the latter island. The ancestral range estimation also suggests that *Rafflesia* colonized the Philippines once and that this involved dispersal and founder-event speciation from Borneo to Panay. From there, *R. speciosa* expanded its range into Negros. A single founder-event speciation from Panay to mainland Luzon resulted in the further expansion of the distribution range of *Rafflesia* in the Philippines. Our results suggest that the Bicol peninsula of Luzon was colonized two times from mainland Luzon: once by the ancestor of *R. lagascae* s.s. and the Mt. Labo population of *R. lagascae* through range expansion, and once involving founder-event speciation resulting in *R. baletei*. A single founder-event speciation from mainland Luzon to Mindanao possibly explains the presence of *Rafflesia* on the latter island. Finally, the presence of *R. manillana* on Samar might be best explained by founder-event speciation from mainland Luzon to Samar.

4. Discussion

4.1. Phylogenetic patterns and relationships

Two previous phylogenetic studies featured Philippine *Rafflesia* species. Barkman et al. (2008) used the same four DNA regions that we selected for our study (*atp*6, *mat*R, *nad*1 B-C, and ITS) and included sequences of *R. lagascae* (identified as *R. manillana*) and *R. speciosa* in addition to 13 Sundaic species. Bendiksby et al. (2010) expanded this data set with additional species (including two more Philippine species) and sequences of a putative plastid 16S region. Our results confirm the reciprocal monophyly of Philippine and Sundaic *Rafflesia* as suggested by these two prior studies, but provide a somewhat different hypothesis regarding the relationships among the *Rafflesia* species groups from Borneo, the Malay Peninsula, and Sumatra and Java than those of Bendiksby et al. (2010). All three studies (i.e. Barkman et al., 2008; Bendiksby et al., 2010; present study) resolve a Malay Peninsula clade and a clade composed of the species from Sumatra and Java. For the Bornean species, Bendiksby et al. (2010) reported a poorly-supported clade, whereas our results and those of Barkman et al. (2008) suggest that these species instead form a paraphyletic group (Figs. 2 and 4). In addition, a sister group relationship between the Borneo and Sumatra/Java clades received a high posterior probability (0.97) in the phylogeny presented by Bendiksby et al. (2010: Fig. 3), but was not recovered by Barkman et al. (2008) nor in our present study, which instead suggest a sister group relationship between the Sumatra/Java clade and the Malay Peninsula clade (Figs. 2 and 4). This relationship is well supported in the phylogeny obtained by Barkman et al. (2008; 0.96 PP), but poorly supported in our phylogeny (0.76 PP, < 50% BS). A high posterior probability was retrieved for a clade composed of *R. tuanmudae* from Borneo and the *Rafflesia* species from Sumatra, Java, and the Malay Peninsula (1.00 PP). These conflicting results signal the need for further study into the evolutionary history of Sundaic *Rafflesia*. Moreover, several species from this region have not been included in a molecular phylogenetic study, including some recently described ones.

As previously demonstrated for Sundaic *Rafflesia* (Barkman et al., 2008; Bendiksby et al., 2010), flower size is generally a poor indicator of the evolutionary relationships among *Rafflesia* species. Similarly, within the Philippine clade closely related species can be remarkably different in flower diameter. For example, *R. schadenbergiana* is the species with the largest flowers in the Philippines (52–80 cm diam; Barcelona et al., 2008, 2009a). It is, however, most closely related to one of the species with the smallest flowers (*R. verrucosa*: 14.5–15 cm diam; Balete et al., 2010) and one with medium-sized flowers (*R. mira*: 45–60 cm diam; Fernando and Ong, 2005; Barcelona et al., 2009a). Barkman et al. (2008) and Bendiksby et al. (2010) further noted that it is not uncommon for sympatric *Rafflesia* species to have quite different

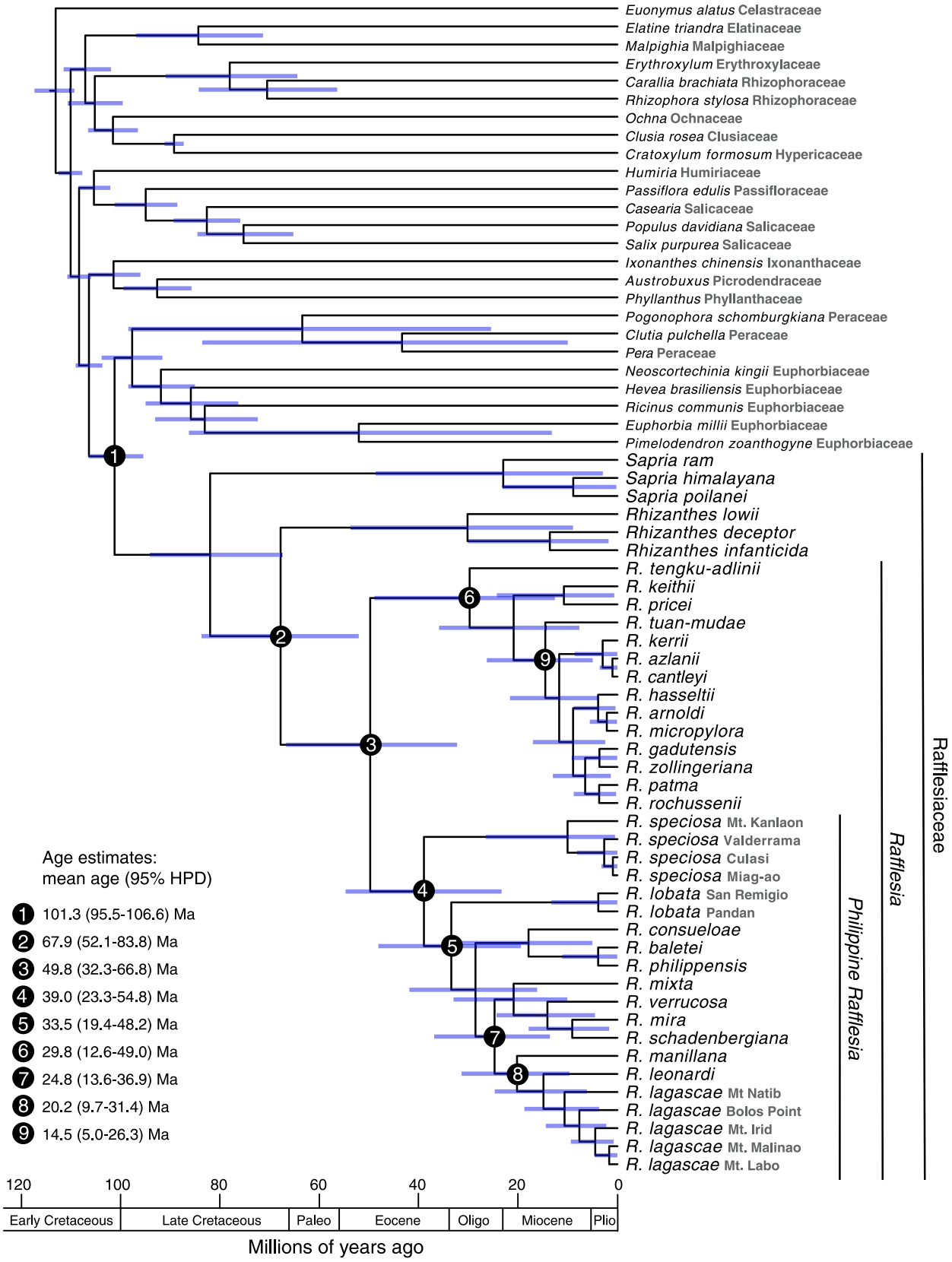

Fig. 3. BEAST maximum clade credibility tree of the combined *atp*6, *mat*R, *nad*1 B-C and ITS data set, using a relaxed-clock and a Yule model. Bars indicate 95% highest posterior density (HPD) intervals. Numbered nodes are discussed in the text.

Table 1
Results of divergence time estimations in BEAST for the combined *atp6*, *matR*, *nad1* B-C and ITS data set, using relaxed-clock Yule and Birth-Death models and a strict clock-like model. Results reported in Bendiksby et al. (2010) and those of an analysis of their data set using a relaxed-clock model are provided for comparison.

Clade	Present study (mean age, 95% HPD)			Bendiksby et al. (2010) (mean age, 95% HPD)	Bendiksby et al. (2010) data set (mean age, 95% HPD)
	Relaxed log normal clock, Yule	Relaxed log normal clock, Birth-Death	Strict clock, Yule		Relaxed log normal clock, Yule
Stem Rafflesiaceae	101.3 (95.5–106.6)	101.2 (95.4–106.6)	108.4 (105.9–110.9)	95.02 (83.14–109.47)	79.85 (77.99–82.21)
Crown Rafflesiaceae	82.1 (67.5–94.2)	79.9 (65.0–93.6)	84.1 (79.5–88.7)	81.67 (69.47–95.89)	62.63 (45.99–76.56)
Stem *Rafflesia*	67.9 (52.1–83.8)	64.5 (47.2–81.0)	75.0 (69.5–80.1)	73.19 (60.76–86.63)	47.72 (29.69–65.48)
Crown *Rafflesia*	49.8 (32.3–66.8)	45.1 (29.0–62.4)	17.1 (14.0–20.3)	11.82 (9.23–15.06)	33.33 (15.74–51.64)
Crown non-Philippine *Rafflesia*	29.8 (12.6–49.0)	25.6 (9.8–43.9)	7.5 (5.1–10.1)	4.25 (4.6–8.43)	22.65 (8.41–38.74)
Crown Philippine *Rafflesia*	39.0 (23.3–54.8)	34.1 (20.2–50.1)	10.6 (8.5–12.8)	7.17 (5.2–9.31)	19.61 (5.55–35.72)
Crown MRCA *R. lobata* & *R. lagascae*	33.5 (19.4–48.2)	29.0 (16.8–43.0)	9.5 (7.7–11.3)	6.46 (4.6–8.43)	13.28 (2.7–26.2)
Crown MRCA *R. consueloae* & *R. lagascae*	28.6 (16.2–41.9)	24.6 (13.8–36.9)	8.4 (6.8–10.1)		
Crown MRCA *R. consueloae* & *R. philippensis*	17.9 (5.1–31.5)	15.6 (4.4–27.9)	6.6 (4.9–8.4)		
Crown MRCA *R. baletei* & *R. philippensis*	3.9 (0–11.1)	3.3 (0–9.2)	0.9 (0.1–1.9)		
Crown MRCA *R. mixta* & *R. lagascae*	24.8 (13.6–36.9)	21.1 (11.2–32.1)	7.6 (6.0–9.1)		
Crown MRCA *R. mixta* & *R. schadenbergiana*	20.9 (10.1–33.0)	17.8 (7.9–28.4)	7.1 (5.5–8.7)		
Crown MRCA *R. verrucosa* & *R. schadenbergiana*	14.1 (4.5–24.4)	11.9 (3.6–20.8)	5.4 (3.9–7.0)		
Crown MRCA *R. mira* & *R. schadenbergiana*	9.1 (1.7–17.9)	7.7 (1.6–15.2)	4.5 (3.0–6.0)		
Crown MRCA *R. manillana* & *R. lagascae*	20.2 (9.7–31.4)	17.2 (8.1–27.3)	6.3 (4.6–8.0)		
Crown MRCA *R. leonardi* & *R. lagascae*	14.9 (6.1–24.7)	12.6 (5.0–21.1)	4.5 (3.0–6.0)		

flower sizes. Because this might prevent successful pollination (by necrophagous and coprophagous flies; e.g., Beaman et al., 1988; Bänziger, 1991; Wee et al., 2018) between flowers of recently diverged species due to pollinator incompatibility, they suggested that it is the result of natural selection for character displacement as a way to avoid gamete wastage and hybridization. Our results could be interpreted as providing additional support for character displacement. For example, *R. lagascae* and *R. leonardi* are each other's closest relatives, are sympatric in two areas, and have different flower sizes (14–23 vs. 25.5–50 cm diam, respectively; Barcelona et al., 2011: note that the species referred to here as *R. manillana* is now *R. lagascae*). Specimens of both *R. lagascae* and *R. consueloae* examined by the last author (CENRO Cabanatuan-Conservation & Development s.n., 19 Jan. 2018, Nueva Ecija Prov., Laur Municipality, Barangay San Vicente, Mt. Kemalugong, PNH) were reportedly collected 5–10 m away from each other (Fig. 1), the latter considerably smaller (6.6–12.7 cm diam; Galindon et al., 2016) than the former. Furthermore, *R. lobata* and *R. speciosa* are sympatric in parts of Panay and are notably different in flower size (11–21 vs. 45–56 cm diam; Barcelona et al., 2011). However, not all sympatric species pairs are different in flower size. For example, *R. lagascae* (14–23 cm diam) is sympatric with *R. philippensis* (17.5–27(–32) cm diam; Barcelona et al., 2007: identified as *R. banahaw* Barcelona, Pelser & Cajano, Madulid et al., 2007: identified as *R. banahawensis* Madulid, Villariba & Agoo) on Mt. Banahaw (Pelser et al., 2013) and Rizal Province (J.M. Agcaoili, J.B. Calinog & J. Matienzo pers. comm.; Fig. 1). The former species also co-occurs with *R. baletei* (9–22 cm diam; Barcelona et al., 2006) in Mt. Asog (Mt. Iriga) in the Bicol peninsula (D. Bagacina, pers. comm.; Fig. 1). Because the sympatric species in these areas belong to different subclades of Philippine *Rafflesia*, and are therefore relatively distantly related (Fig. 2), the absence of character displacement in flower size could be explained by assuming that intersterility due to the accumulation of genetic differences between these species evolved before they became sympatric. It is, however, also possible that their sympatry is too recent for character displacement to be noticeable, or that displacement has evolved in characters other than flower size.

Bendiksby et al. (2010) concluded that in addition to flower size, other morphological characters also show high levels of homoplasy in *Rafflesia*: flower color, presence of white warts, and the number of processes on the disk. Likewise, the patterns of diversity in the morphology of the ramenta (bristle-like structures that cover the inner parts of the floral tube; e.g., Meijer, 1997; Nais, 2001) revealed in a later study (Susatya et al., 2017) are incongruent with the phylogenetic patterns revealed by Barkman et al. (2008), Bendiksby et al. (2010), and in the present study. Although we did not study this in detail for Philippine *Rafflesia*, the phylogenetic relationships among Philippine species obtained from our expanded data set indeed failed to reveal obvious morphological synapomorphies for most Philippine clades; however, a few subtle patterns are discernible. The diaphragms (tissue surrounding the opening of the floral tube; e.g., Meijer, 1997; Nais, 2001) of *R. baletei*, *R. consueloae*, and *R. philippensis* (and also *R. aurantia*, not sampled in this study) are relatively rugose compared to most other Philippine *Rafflesia* species. In addition, the apertures of their flowers are relatively small (i.e. diaphragm/aperture ratio > 2; data from Barcelona et al., 2009a and pers. obs.), although some flowers of *R. manillana*, *R. schadenbergiana*, *R. speciosa*, and *R. verrucosa* have a similar diaphragm/aperture ratio. In contrast, the species pair *R. lagascae* and *R. leonardi* is characterized by having very wide apertures (diaphragm/aperture ratio < 1.5), a character otherwise only found in *R. lobata*.

The identity of the *Tetrastigma* species that each Philippine *Rafflesia* species parasitizes (i.e. their host range) is also a poor indicator of their evolutionary relationships. All five *Tetrastigma* species that are hosts of more than one *Rafflesia* species (Pelser et al., 2016) are parasitized by species that are not each other's closest relatives (Fig. 2). Despite this, some closely related *Rafflesia* species share the same host species.

Table 2
Results of BioGeoBEARS model testing. AIC and AICc comparisons of different models of biogeographical range evolution and estimates for: d (dispersal), e (extinction) and j (weight of jump dispersal/founder speciation).

Model	No. of parameters	LnL	d	e	j	AIC	AIC weight	AICc	AICc weight
DEC	2	−69.98	0.0014	1.00E-12	0	144	0.0044	144.4	0.0055
DEC + J	3	−63.57	0.0006	1.00E-12	0.02	133.1	1	134	0.99
DIVALIKE	2	−99.92	0.01	0.01	0	203.8	4.40E-16	204.2	5.50E-16
DIVALIKE + J	3	−99.94	0.01	0.01	0.0001	205.9	1.60E-16	206.7	1.60E-16
BAYAREALIKE	2	−126.8	0.01	0.01	0	257.5	9.70E-28	257.9	1.20E-27
BAYAREALIKE + J	3	−126.2	0.01	0.01	0.0001	258.4	6.30E-28	259.2	6.30E-28

Table 3
Results of BioGeoBEARS biogeographical stochastic modeling using the DEC + J model.

Mode	%	Type	Mean (SD)	%
Within-area speciation	61.4	Speciation (y)	18.84 (1.64)	51.8
		Subset speciation (s)	3.5 (1.97)	9.6
Dispersal	31.3	Jump dispersal/founder events (j)	7.05 (1.29)	19.4
		Range expansions (d)	4.34 (0.93)	11.9
		Range contractions (e)	0	0.0
Vicariance	7.2	Vicariance (v)	2.61 (1.05)	7.2
Total			36.34 (0.93)	100.0

Rafflesia baletei, *R. consueloae,* and *R. philippensis* parasitize *T.* cf. *magnum* Merr., and *R. lagascae*, *R. leonardi*, and *R. manillana* all parasitize *T. loheri* Gagnep. (Pelser et al., 2016; this study). In combination with the findings of studies that examined patterns of host-specificity and host race formation (Pelser et al., 2016, 2018), our results suggest that cospeciation might not have occurred in the diversification of Philippine *Rafflesia* and *Tetrastigma*.

4.2. Divergence time estimations

The results of our divergence time estimations should be considered with care. We had to rely on calibration points external to Rafflesiaceae, because there are no known fossils of this family (Bendiksby et al., 2010). In addition, although we assumed that the phylogenetic patterns resolved in this study are correct, not all clades received high support values. Furthermore, parasitic plants generally show elevated substitution rates compared to non-parasitic lineages (Bromham et al., 2013) and this has also been demonstrated for Rafflesiaceae (e.g., Duff & Nickrent, 1997; Nickrent et al., 2004). These differences in rates of molecular evolution between Rafflesiaceae and autotrophic lineages in our analyses might have resulted in errors in our estimated divergence times, despite the use of a relaxed-clock approach.

Our estimates of divergence dates of *Rafflesia* species and lineages are older than those obtained in previous studies (Barkman et al., 2008; Bendiksby et al., 2010; Fig. 3, Table 1). Barkman et al. (2008) estimated that the crown age of *Rafflesia* is c. 12 Ma, with most speciation events taking place in the most recent 2 Ma. Those age estimates were obtained from analyses that assumed a strict molecular clock, although Barkman et al. (2008) reported that using a relaxed clock provided similar estimates of divergence times. Bendiksby et al. (2010) did not specify whether a strict or a relaxed clock was used for their BEAST analyses of the expanded Barkman et al. (2008) data set, but they arrived at a similar estimate for the crown age of *Rafflesia*: 11.82 Ma (95% HPD 9.23–15.06 Ma). In addition, they estimated that the Philippine *Rafflesia* clade started diversifying 5.2–9.31 Ma (mean 7.17 Ma), followed by the onset of diversification of Sundaic *Rafflesia* between 4.6 and 8.43 Ma (mean 4.25 Ma). In contrast, our results suggest that the crown age of *Rafflesia* is between 32.3 and 66.8 Ma (mean 49.8 Ma), that the first speciation event for the Philippine species included in our analyses took place between 23.3 and 54.8 Ma (mean 39.0 Ma), and that the crown age of Sundaic *Rafflesia* is 12.6–49.0 Ma (mean 29.8 Ma; Fig. 3, Table 1). These results were obtained when a relaxed lognormal clock and a Yule tree prior were selected, following the rejection of the strict clock-like hypothesis that resulted from a stepping-stone analysis. Similar divergence dates were estimated when a Birth-Death tree prior was assumed (Table 1). A BEAST analysis of our data set using a strict clock model resulted in age estimates that were notably more similar to those obtained by Bendiksby et al. (2010; Table 1). Furthermore, a BEAST analysis of the Bendiksby et al. (2010) data set using a relaxed lognormal clock and Yule tree prior resulted in markedly older age estimates for the diversification of *Rafflesia* (Table 1). It is therefore likely that the large differences in age estimates between these studies are explained by different assumptions regarding the presence of a constant speciation rate in Rafflesiaceae. Because the results of our stepping-stone analysis of the Bendiksby et al. (2010) data set strongly rejected the strict clock hypothesis (mean marginal likelihood of −14390.47 for the strict clock hypothesis vs. −14360.29 for the unconstrained hypothesis; $2\ln BF_{12} = 60.36$), we believe that the assumptions we have used result in more accurate age estimates. If this is correct, our results suggest that the diversification of Rafflesiaceae happened more gradually than what was previously assumed. That assumption involved a long period of time without net diversification followed by an explosive increase in diversification in the last 12 Ma (Bendiksby et al., 2010). This also implies that the rates of flower size evolution in *Rafflesia* might be slower than those reported by Barkman et al. (2008).

4.3. Biogeography

The results of our BioGeoBEARS analyses should be interpreted with care, because not all *Rafflesia* clades are supported with high posterior probabilities and bootstrap support values (Fig. 2). Under the assumption that the phylogenetic relationships among *Rafflesia* species that were resolved in our study are correct, the BioGeoBEARS analyses provide a somewhat different hypothesis about the early biogeographic history of *Rafflesia* than that proposed by Bendiksby et al. (2010). Although only supported by a relative probability < 50% (Fig. 4), our results indicate that Borneo has the highest probability of being the ancestral range of *Rafflesia*. This contrasts with the results of Bendiksby et al. (2010), which instead suggested that the ancestor of *Rafflesia* was widespread throughout its present distribution range. They postulated that subsequent vicariance was responsible for the evolution of the four geographically distinct clades that were resolved in their phylogenetic analyses (i.e. Borneo, Java and Sumatra, Malay Peninsula, Philippines). Our results instead support a more significant role for dispersal in the early diversification of *Rafflesia* (Table 3) and point at the possibility that *Rafflesia* first dispersed from Borneo into the Philippines (32.3–66.8 Ma) and later (5.0–26.3 Ma) from Borneo into either the Malay Peninsula and subsequently to Java and Sumatra, or vice versa (Fig. 4). The indication that the ancestral distribution range of *Rafflesia* might have been considerably smaller than previously proposed (Bendiksby et al., 2010) is intuitively appealing because all extant *Rafflesia* species are only found in at most two islands of the Malesian region.

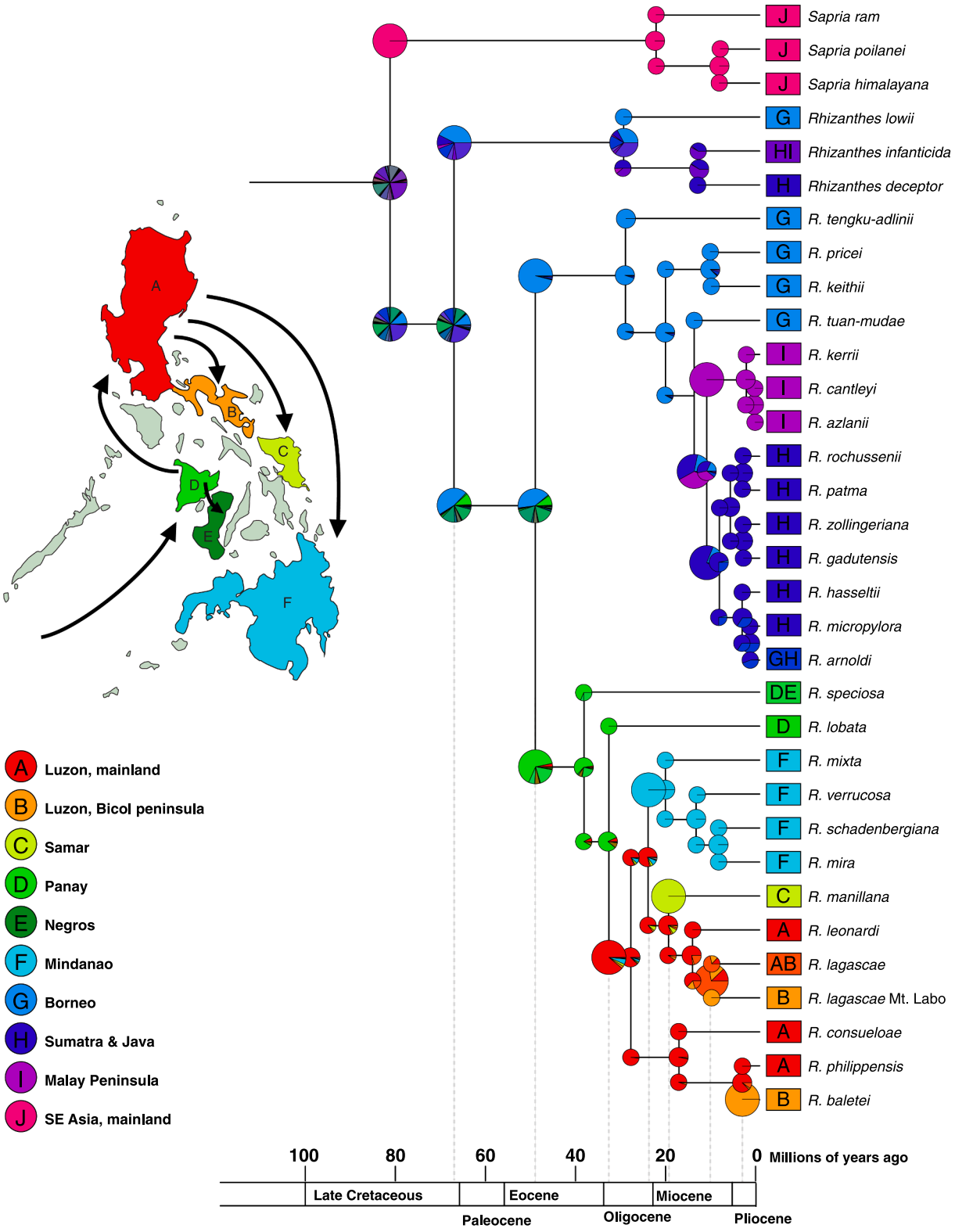

Fig. 4. Ancestral range estimation by BioGeoBEARS (DEC + J model; 4 areas max., d = 6e−04, e = 0, j = 0.02, LnL = −63.57). Pie diagram circles show the relative probabilities of ancestral range hypotheses. Larger circles indicate biogeographic events discussed in the text.

Under the assumption that our older divergence time estimates are correct, the Philippines might have been colonized by *Rafflesia* at a time when, according to a plate tectonic model resulting from a synthesis of the literature on the Cenozoic development of Southeast Asia by Hall (2002), fragments of this archipelago were connected via the Sulu-Cagayan Arc to Borneo, and positioned closer to that island than they are at present (i.e. c. 45–50 Ma; Hall, 2002). These fragments potentially included those that would later form Panay Island (Hall, 2002), which might be where *Rafflesia* first diversified in the Philippines (Fig. 4). From Panay, we hypothesize that *Rafflesia* dispersed to what is now mainland Luzon 33.5 Ma (95% HPD 19.4–48.2). About 24.8 Ma (95% HPD 13.6–36.9), the genus possibly dispersed from Luzon to Mindanao

(Fig. 4). At this time the land mass that forms present-day central and eastern Mindanao was distant from Luzon (in the Molucca Sea). However, the western portion of present-day Mindanao, the Zamboanga Peninsula, was part of the Sulu Arc that was proximal to Luzon c. 20 Ma. By the end of the Miocene (5.3 Ma), central and eastern Mindanao and the Zamboanga region were close and later the terranes joined. However, the BioGeoBEARS tree indicates that Mindanao *Rafflesia* diversified c. 20 Ma, which would require that *Rafflesia* spread from the Zamboanga Peninsula to other parts of Mindanao. And because *Rafflesia* has not been reported from the Zamboanga Peninsula (Fig. 1), either these parasites remain to be discovered in this poorly explored area, or the ancestral populations have gone extinct.

According to our estimations, *Rafflesia* also spread from Luzon to Samar. This might have occurred 20.2 Ma (95% HPD 9.7–31.4). However, like the larger part of Mindanao, Samar was at that time located relatively far to the southeast of Luzon (Hall, 2002). The terranes that compose the Bicol peninsula of Luzon are also among those that have a Southern Hemisphere origin. The Bicol peninsula possibly first connected with mainland Luzon 5–10 Ma (Hall, 2002) and was subsequently colonized twice by *Rafflesia* lineages from mainland Luzon (Fig. 4). The Philippine archipelago has an extremely complex geological history and is composed of an uncertain number of terranes of uncertain origin and relationships (Hall 2002). In addition to Hall's (2002) hypothesis of the early geological history of the Philippines, markedly different hypotheses have been proposed. For example, the results of geological modeling by Zahirovic et al. (2016) suggest that a much larger part of the Philippines has its origin in the Southern Hemisphere. Although all biogeographic hypotheses involve some speculation, the lack of consensus regarding the tectonic and geological history of the Philippines makes any detailed interpretation of our data problematic at this time.

One of the two Luzon lineages that dispersed into the Bicol peninsula is *R. lagascae*. The genetic diversity and structure of this species were previously examined using microsatellite data (Pelser et al., 2017), and that study revealed that the *R. lagascae* population of Mt. Labo is genetically distinct from all other sampled *R. lagascae* populations, including the population of Mt. Malinao, which is also located on the Bicol peninsula of Luzon (Fig. 1). The results of the analyses presented here suggest that the two populations from Bicol (Mt. Labo and Mt. Malinao) are closely related relative to the populations from mainland Luzon (Fig. 2). These patterns suggest that the Mt. Labo population has been genetically isolated from the Mt. Malinao and other *R. lagascae* populations for a relatively long time, but it is presently unclear which factors are responsible for this.

Our biogeographic results support the hypothesis that the pronounced pattern of island endemism of *Rafflesia* is a result of poor inter-island dispersal abilities because such events appear to have been very rare in the evolutionary history of *Rafflesia*. This is especially evident in the Philippines. Even if the current distribution pattern of Philippine *Rafflesia* is exclusively the result of dispersal between the areas that form the present-day islands (i.e. a total absence of vicariance), each of these islands was only colonized once (Fig. 4). The absence of *Rafflesia* on several large islands of the Philippine archipelago (e.g., Bohol, Cebu, Mindoro, Palawan; Fig. 1) provides further support of poor inter-island dispersal abilities, although *Rafflesia* may yet be discovered on these islands, or there may have been local extinctions. Both alternative explanations are certainly feasible: new species and populations of Philippine *Rafflesia* are still being discovered (e.g., Barcelona et al., 2014; Galindon et al., 2016), and previous and on-going large-scale destruction and degradation of the tropical rainforest habitat of *Rafflesia* has, without doubt, resulted in local extinction (Pelser et al., 2017, 2018). Inter-island dispersal also appears to have been rare in the other parts of the distributional range of *Rafflesia* (Fig. 4). Because we focused our study on Philippine *Rafflesia* and did not have access to DNA from Sundaic species, we did not examine in detail the biogeographical patterns of *Rafflesia* outside of the Philippines. However, only five of the Sundaic species are reported from more than one island (*R. arnoldi* (Sumatra and Borneo, but see below), *R. cantleyi* (Peninsular Malaysia and Tioman Island), *R. gadutensis* (Sumatra and Mursala Island), and *R. patma* and *R. rochussenii* (Sumatra and Java); Meijer, 1997; Hidayati et al., 2000; Nais, 2001; Mahyuni et al., 2015; Mursidawati et al., 2015). Future biogeographical studies with a larger number of *Rafflesia* species are therefore not likely to increase the number of inferred dispersal events substantially.

In addition to the low number of inferred dispersal events, the finding that these events usually resulted in founder-event speciation (instead of range expansion) also provides evidence in support of poor inter-island dispersal. Range expansion between Philippine islands was only inferred for *R. speciosa* (Fig. 4). *Rafflesia arnoldi* is the only Sundaic species for which the data suggest inter-island range expansion. This species is reported from both Sumatra and Borneo (Meijer, 1997; Nais, 2001), but very little is known about *R. arnoldi* in Borneo and, to our knowledge, its presence on that island has not been conclusively demonstrated. Although relatively distantly related (Barkman et al., 2008; Bendiksby et al., 2010; Fig. 2), *R. arnoldi* is morphologically similar to *R. tuan-mudae* (Coomans de Ruiter, 1933; Meijer, 1958) which is found in southwest Borneo and has been confused with *R. arnoldi* in the past (Meijer, 1997; Susatya et al., 2017).

5. Conclusion

The results of our phylogenetic and biogeographic study of Philippine *Rafflesia* confirm those of previous studies focused on non-Philippine *Rafflesia* species (Barkman et al., 2008; Bendiksby et al., 2010) in finding pronounced biogeographic structure in our data sets. The biogeographic patterns suggest that dispersal between islands has been relatively uncommon, and therefore indicate that the high island endemism of *Rafflesia* is a result of poor inter-island dispersal abilities. It is possible that *Rafflesia* is dispersal-limited and that this is related to seed dispersal by ants, but more research into the seed dispersal of *Rafflesia* species is needed. Our findings further contribute to the knowledge of the diversification of *Rafflesia* by indicating that its ancestral range might have been in Borneo, and that its lineages and species evolved considerably earlier and more gradually than previously assumed (Barkman et al., 2008; Bendiksby et al., 2010).

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Appendix A. Supplementary material

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