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Report

Accelerated Rates of Floral Evolution at the Upper Size Limit for Flowers

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Summary

Evolutionary theory explains phenotypic change as the result of natural selection, with constraint limiting the direction, magnitude, and rate of response [1]. Constraint is particularly likely to govern evolutionary change when a trait is at perceived upper or lower limits. Macroevolutionary rates of floral-size change are unknown for any angiosperm family, but it is predicted that rates should be diminished near the upper size limit of flowers, as has been shown for mammal body mass [2]. Our molecular results show that rates of floral-size evolution have been extremely rapid in the endoholoparasite *Rafflesia*, which contains the world's largest flowers [3]. These data provide the first estimates of macroevolutionary rates of floral-size change and indicate that in this lineage, floral diameter increased by an average of 20 cm (and up to 90 cm)/million years. In contrast to our expectations, it appears that the magnitude and rate of floral-size increase is greater for lineages with larger flowered ancestors. This study suggests that constraints on rates of floral-size evolution may not be limiting in *Rafflesia*, reinforcing results of artificial- and natural-selection studies in other plants that demonstrated the potential for rapid size changes [4–6].

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Results and Discussion

Rafflesia Species Exhibit Low Levels of Molecular Divergence

Rafflesiaceae are composed of three genera of bizarre endoholoparasitic flowering plants, *Rafflesia*, *Rhizanthes*, and *Sapria*, that are largely restricted to Southeast Asia [3, 7] and grow as strands of cells embedded within host stem and root tissues, emerging only as flowers during sexual reproduction. *Rafflesia* produces the largest flower in the world (ca. 100 cm), and within the genus, floral-size variation spans nearly one order of magnitude (11–100+ cm) [3, 8]. In spite of this extensive floral-size variation, an unexpected finding was revealed from Bayesian-estimated divergence times and phylogenetic relationships [9]: species exhibit little DNA-sequence variation, as indicated by the very short estimated branch lengths shown in Figures 1 and 2. In fact, the Peninsular Malaysian species *R. kerrii* and *R. cantleyi* are identical in sequence except for one insertion in the *nad1 B-C* intron; yet their flowers differ in diameter by 20 cm [3]. This analysis suggests that most closely related *Rafflesia* species are found in the same geographic region and that the genus is ca. 12 million years (My) old (Figure 2). However, most divergences among species are estimated to have occurred within the last 1–2 My, and in several instances, speciation appears to have occurred within the last 600,000 years, in both Indonesia and Peninsular Malaysia (Figure 2). The young-age estimates for speciation events within *Rafflesia* are corroborated through alternative methodologies that do not assume a strict molecular clock [10] (data not shown). The divergence-time estimates for *Rafflesia* are probably not affected by differences in generation time either, because various species appear to be similar [3], although *Rhizanthes* and *Sapria* have not been studied. Repeating our methods on independent collections and extractions produced the same results; thus, we are confident that the high levels of similarity among species are not due to contamination. The low level of molecular variation within *Rafflesia* is surprising, because both the nuclear rDNA internal transcribed spacer (ITS) region and the *nad1 B/C* intron are usually variable among closely related species [11–14]. The fact that the ITS is so similar among these divergent species is particularly surprising, because it is linked to the nuclear rDNA small subunit of *Rafflesia*, which is the most rapidly evolving in all plants and exhibits divergences ca. five times higher than those of nonparasitic species [15, 16]. The same is also true for Rafflesiaceae mtDNA, which exhibits divergences ca. two times higher than those of nonparasitic plants [16–18]. Low levels of molecular divergence among recently evolved, morphologically distinctive species have also been reported in several other lineages [19–22].

Rates of Floral-Size Evolution Are Remarkably High within *Rafflesia*

The flowers of *Rafflesia* are thought to mimic rotting flesh and are 10–100 times larger in diameter than those of most other flowering-plant genera. Because they represent the upper limit of floral size, we expected that large flowers would have evolved over long periods of time and probably only once

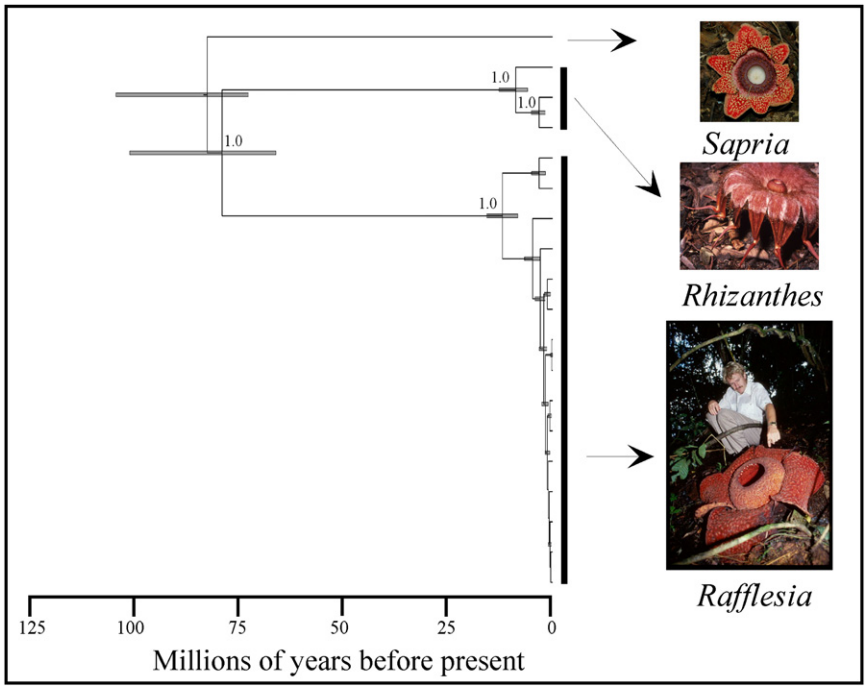

Figure 1. Chronogram Showing Relationships and Divergence Times among the Three Genera of Rafflesiaceae

In spite of considerable variation in floral size within *Rafflesia*, very little molecular variation is observed, as indicated by the short branch lengths. Grey bars at nodes represent the 95% highest posterior density interval of divergence-time estimates. Posterior probabilities for all nodes except those within *Rafflesia* are shown. Details of divergence times and relationships with posterior probabilities for clades within *Rafflesia* are shown in Figure 2. Images for representative species of the three genera are shown.

during the history of the genus. Contrary to expectations, these large flowers appear to have evolved rapidly, recently, and repeatedly (Figure 2). A comparison of two models of trait evolution [23] indicates that a model allowing the rate of floral-size evolution in Rafflesiaceae to accelerate over time is a better fit to the data than is one implying gradual and constant change (likelihood-ratio test = 9.5, 1 df, $p = 0.002$). To more precisely determine the timing of this acceleration, we investigated whether a model allowing the rate of floral-size evolution to be different between the *Rafflesia* clade, (which has large floral sizes that vary substantially in diameter) and the rest of the family (which have smaller flowers [< 25 cm] that do not vary much in size) is better than a model assigning one rate to the whole tree (Figure 1) [24]. In this case, the best-fit model of floral-size evolution assigns to the *Rafflesia* clade a rate that is twenty times higher than the separate rate estimated for the rest of the family (difference between the Akaike Information Criterion of the two models being compared = 5.9).

To further investigate the history of floral-diameter change in *Rafflesia*, we estimated ancestral states through a novel Bayesian approach [25]. An advantage of the Bayesian approach implemented here is that uncertainty of ancestral states is estimated (Figure 2 panels) and can be explicitly incorporated into subsequent analyses. These estimates show that the evolutionary history of the genus is mostly one of small floral-size changes, until the last 1–2 My (Figure 2). Within this window of time, ancestors ranged from ca. 30–40 cm in diameter on average (after back log transformation), persisted throughout numerous speciation events, over both long and short periods of time, then gave rise to species much larger and smaller in diameter. Remarkably, it appears that large flowers (> 60 cm) have evolved multiple times from smaller-flowered ancestors in Indonesia, Borneo, and Peninsular Malaysia (Figure 2). In fact, a statistical test [26] indicates that trees constraining the large-flowered species (> 60 cm diameter) to be monophyletic at node A, D, or F are significantly worse than the optimal tree shown in Figure 2 (Shimodaira-Hasegawa Test significant at $p < 0.005$ in all cases).

Estimates of net rates of floral-size evolution were obtained by calculating the amount of flower-size change occurring during the elapsed time shown on each branch in Figure 2. An average overall rate of floral-size change of 4 cm/My is estimated to have occurred in *Rafflesia*. Interestingly, ancestral-size estimates reveal that there have been many decreases (avg. 11 cm/My) and increases (avg. 20 cm/My) in floral size over the 12 My of *Rafflesia* evolutionary history. The maximum average rate of floral-size increase is calculated for the *R. arnoldii* and *R. kerrii* branches that have evolved at 58 cm and 90 cm/My, respectively, since diverging from their ancestors. A previous study of Rafflesiaceae suggested that a large increase in the rate of floral-size evolution occurred in the lineage separating the family from the rest of Malpighiales and that there was no evidence for rate differences among *Rafflesia*, *Rhizanthes*, and *Sapria* [27]. Perhaps because the branching relationships among Rafflesiaceae species were not estimated in that study, the large shift in evolutionary rate reported here for *Rafflesia* was not detected. Furthermore, although a large change in floral size occurred along the lineage leading to the ancestor of Rafflesiaceae, it occurred over 46 My [27]; a net rate of ca. 0.41 cm/My. Clearly, the more recent evolutionary changes in *Rafflesia* are very high and indicate that the evolutionary history of floral-size change in the genus is dynamic. In this context, it is fascinating that a wide range of floral sizes has evolved without much apparent concomitant evolutionary change on the part of the host, because a number of very differently sized *Rafflesia* species parasitize the same host species [3, 8]. However, *Rafflesia* does appear to exhibit typical life-history tradeoffs, with large-flowered species generally producing fewer flowers than small-flowered species [3]. Other parasites do not produce particularly large flowers, so it does not appear that the heterotrophic lifestyle of parasitic plants alone accounts for the rapid evolution of large flowers. Instead, it may be that the carrion-fly-pollination syndrome results in selection for large floral-display sizes, because other genera with some of the largest flowers or inflorescences in the world (*Aristolochia*, *Stapelia*, and various Araceae, including *Amorphophallus*) are also sapromyophilous.

Large-Flowered Ancestors Have Given Rise to Descendants with Even Larger Flowers

Because the flowers of *Rafflesia* represent the upper limit of floral size, we predicted that rates of change should be

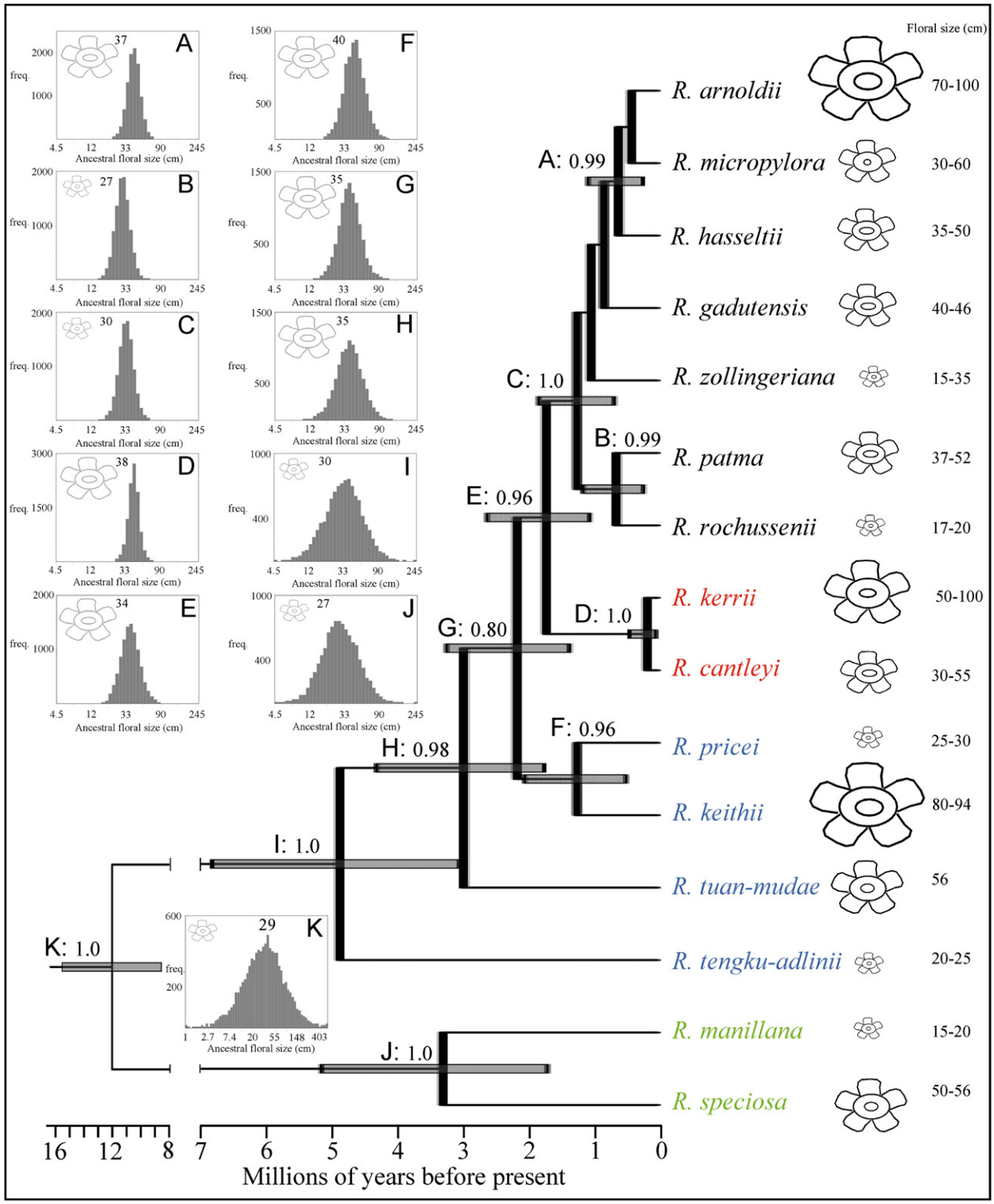

Figure 2. Expanded Chronogram Showing Phylogenetic Relationships and Divergence Times for the *Rafflesia* Clade Shown in Figure 1

Posterior probabilities of 0.8 or greater are shown at each node. Grey bars at nodes represent the 95% highest posterior density interval of divergence-time estimates with nodes placed at the mean posterior divergence-time estimate. Most speciation events are estimated to have occurred within the last 2 My. Shown in each labeled panel are the posterior distributions of ancestral floral diameters estimated for each labeled node, with mean sizes listed above. Although ancestral states were estimated with ln-transformed data, all sizes shown have been back log transformed. Ancestral floral sizes are inferred to have been more or less static over time, with mean sizes ranging from 27–37 cm for most of the history of the genus, with net changes along each branch less than 7 cm on average. However, within the last 1–2 My, large floral-size increases and decreases have occurred from these intermediate-sized ancestors. Species names shown in black are mostly found in Indonesia (Sumatra and Java), red are found in Peninsular Malaysia and Thailand, blue are restricted to Borneo, and green are endemic to the Philippines. Ranges of floral diameter are shown next to each extant species. Grey floral outlines are ancestral, black are extant.

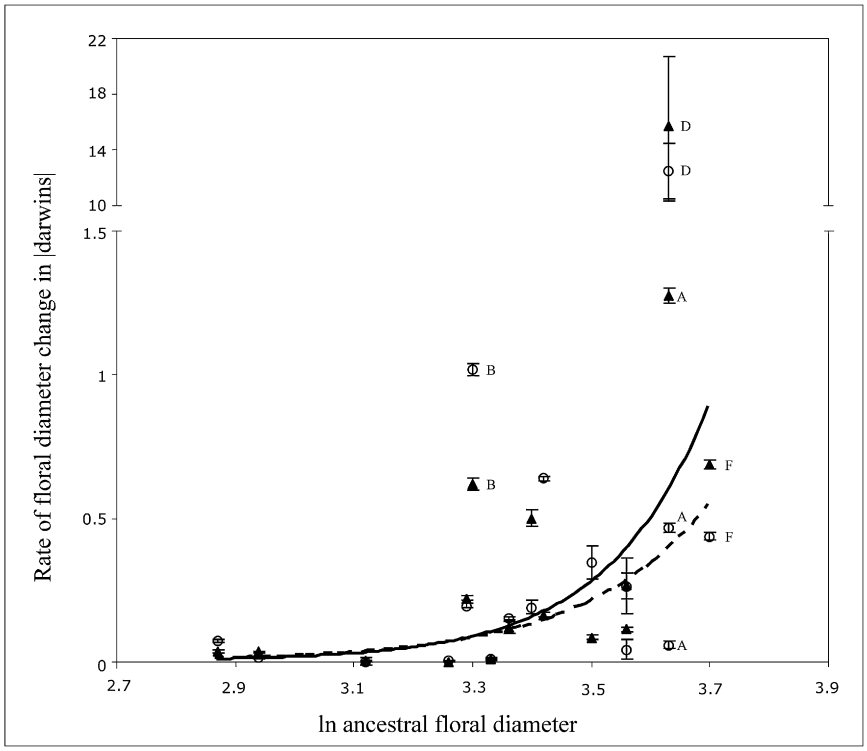

Figure 3. Relationship of Net Rate of Floral-Size Change in Absolute Value of Darwins to log-Transformed Ancestral Floral Diameter for Each Branch in Figures 1 and 2

Mean rates of floral-size increase and decrease, with standard errors, are represented by triangles and circles, respectively. Rates of floral-size change are higher for larger ancestral floral sizes (Spearman's rank correlation coefficient = 0.71, p = 0.002). Rates for branches descending from shared nodes A, B, D, and F are labeled so that their paired, divergent rates of change can be compared. Best-fit exponential trend lines are shown for both floral-size increases (solid) and decreases (dashed). Darwins are calculated with the following formula: ln(descendant floral size) − ln(ancestral floral size) / elapsed time in My. Rates of change for trait values that increase over time are positive, whereas those that decrease are negative.

decreased for large ancestors, as has been demonstrated for mammal body mass [2]. In the case of *Rafflesia* flowers, if constraints limit responses to selection [28], then rates of change toward larger flowers should be low. Rates of floral-diameter evolution were calculated in darwins [29, 30] which utilize log-transformed data, so that comparisons could be made across each branch independently on the tree shown in Figures 1 and 2. Figure 3 (triangles) shows a surprising pattern of the relationship between ancestral flower size and rate of floral-size change: the rate of floral-size enlargement increases as the flower size of the ancestor increases (Spearman's rank correlation coefficient = 0.71, p = 0.002). The relationship of ancestral flower size and floral-size change (instead of change over time) also indicates that larger ancestral flowers have given rise to larger descendants (Figure S1, available online). The apparent relationship between rate of change and ancestral floral size is not necessarily due to the short time intervals over which large magnitudes of change have occurred. If rates of change occurring within the last 0.6–1.6 My—the window of time within which most speciation events have occurred—are compared, the positive relationship between higher net rates of change in darwins and larger ancestral floral size is still observed. Uncertainty in the ancestral-state estimation shown in Figure 2 and use of an Ornstein-Uhlenbeck process [31] to model stabilizing selection did not affect the conclusions drawn, either (Figures S2 and S3, respectively). Thus, *Rafflesia* species appear to be free from constraint, allowing for rapid change even at the apparent upper limit of floral diameter. This raises the possibility that 100 cm may not be the upper limit for flower size in angiosperms.

The deceptive carrion-fly-pollinated flowers of *Rafflesia* are thought to mimic rotting corpses [3, 32]. Thus, the rapid changes reported here could be due to selection favoring the mimicking of differently sized corpses corresponding to carrion-fly species' differing innate preferences for carcass size [33, 34]. In addition to size differences, *Rafflesia* species also differ in pigmentation patterns, wart patterning, and scent, and these characters may also be under selection, because they may indicate alternative stages of decay to which different carrion-fly species may specialize. Alternatively, selection for reproductive isolation by character displacement for avoidance of gamete wastage may be responsible for some of the variation observed, particularly in regions of sympatry [35]. Consistent with this idea is the pattern of floral-size divergence observed for the largely sympatric species pairs that have undergone rapid, simultaneous increases and decreases from intermediately sized ancestors (Figures 2 and 3, nodes A, B, D, and F). In this case, selection could promote flower-size divergence, because small-bodied insects would not effectively pollinate large flowers [32, 36], and vice versa, even though the same flies might visit flowers of all sizes. Experimental manipulations aimed at testing between these hypotheses are needed. In contrast, it does not appear likely that differences in ploidy level or altitudinal distribution could have driven the floral-size changes in this lineage, because chromosome number does not vary among large- and small-flowered species [37] and altitude is not correlated with floral size ($r = -0.12$, $p = 0.7$). Environmental factors, like host-vine physiological state, could contribute to floral variation within and among species, but systematic data for assessing the impact of this variable are lacking.

In conclusion, it should be expected that investigation of the thousands of recently evolved tips of the Tree of Life will reveal many instances of rapid trait diversification. Theoretically, the ability of a lineage to respond adaptively depends on the strength of selection balanced by some level of constraint. Artificial- and natural-selection studies have shown that responses to selection can be quite rapid and provide evidence that, at least in the short term, constraint may not be limiting trait evolution [38, 39]. Furthermore, the implicit notion that rates of change may be decreased near the theoretical upper limit for a trait need not be invoked because, as our data indicate, apparent bounds can be rapidly surpassed.

Experimental Procedures

Taxon and Gene Sampling

To study floral-size evolution in *Rafflesia*, we sampled 15 out of the ca. 20 extant species from throughout *Rafflesia*'s geographic range, as well as representatives of the related genera *Rhizanthes* and *Sapria*. Table S1 shows all taxa sampled, voucher information, and GenBank accession numbers for all sequences used in this study. A total of 6130 aligned nucleotides from the combined nuclear (ITS) and mitochondrial (*matR*, *atp6*, and *nad1 B-C*) markers were compared. All DNA sequences were generated with standard molecular methods. Additional details are provided in Supplemental Data.

Phylogenetic Analyses

BEAST (ver. 1.4.6) [9] was used for estimating the posterior-probability distribution of divergence times at each node in the Rafflesiaceae phylogeny via a most recent common ancestor (MRCA) approach. This Bayesian approach is desirable because phylogenetic uncertainty can be accounted for with the set of topologies contained within the posterior-probability distribution for divergence-time estimation [40].

BayesTraits [25] was used for estimating the posterior-probability distribution of ancestral states. For all species, we scored log-transformed floral diameters according to the minimum reported value for the known range. We obtained 1000 trees from the set of topologies and branch lengths generated by BEAST during divergence-time estimation and used them for ancestral-state estimation via the Markov Chain Monte Carlo (MCMC) approach.

We used the software program Continuous [23, 41] for maximum-likelihood parameter estimation to determine the optimum value of the scaling parameter, delta. This parameter is used to detect whether the rate of trait evolution has been constant or has changed over time. A value of delta significantly greater than 1.0 indicates that the rate of evolution has accelerated over time whereas a value of 1.0 is consistent with a constant rate of change. We next used Brownie [24] to implement a censored approach to compare two rate models. In the first model, a single rate of evolution was applied to the entire tree. In the second model, one rate of evolution was estimated for the *Rafflesia* clade and a second rate was estimated for all other branches in the tree. For these tests we used the topology and mean branch lengths shown in Figure 1. Further details on all phylogenetic analyses are provided in Supplemental Data.

Rate Calculations in Darwins

We used the darwin [29] as a measure of the net rates of evolutionary change along each branch of the Rafflesiaceae phylogeny shown in Figures 1 and 2. This measure is obtained by dividing estimated floral-size change (in log units) along a branch by the estimated elapsed time, in My, along the same branch. To obtain estimates of floral-size change on each branch of the Rafflesiaceae phylogeny, ancestral-state values were subtracted from descendant-state values obtained from the MCMC analysis implemented in BayesTraits to yield a distribution of net change.

Statistics

Prior to determining the relationship between variables, we checked data for bivariate normality. If the data were normal, we used the Pearson product-moment correlation coefficient. If the data were not normal, the Spearman's rank correlation was used. In our specific case of comparing the relationship between darwins and ancestral floral size, we did not log transform the rates to achieve normality, because the numerator was already log transformed (see above).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/19/1508/DC1/.

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