Temperate radiations and dying embers of a tropical past: the diversification of *Viburnum*

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Summary

• We used a near-complete phylogeny for the angiosperm clade *Viburnum* to assess lineage diversification rates, and to examine possible morphological and ecological factors driving radiations.

• Maximum-likelihood and Bayesian approaches identified shifts in diversification rate and possible links to character evolution. We inferred the ancestral environment for *Viburnum* and changes in diversification dynamics associated with subsequent biome shifts.

• *Viburnum* probably diversified in tropical forests of Southeast Asia in the Eocene, with three subsequent radiations in temperate clades during the Miocene. Four traits (purple fruits, extrafloral nectaries, bud scales and toothed leaves) were statistically associated with higher rates of diversification. However, we argue that these traits are unlikely to be driving diversification directly. Instead, two radiations were associated with the occupation of mountainous regions and a third with repeated shifts between colder and warmer temperate forests.

• Early-branching depauperate lineages imply that the rare lowland tropical species are 'dying embers' of once more diverse lineages; net diversification rates in *Viburnum* likely decreased in these tropical environments after the Oligocene. We suggest that 'taxon pulse' dynamics might characterize other temperate plant lineages.

Introduction

Identifying heterogeneity in diversification rates is essential to understanding the evolutionary processes that generate diversity. The continuing development of statistical methods (e.g. Maddison *et al.*, 2007; Alfaro *et al.*, 2009; Rabosky, 2014) and their application in a wide variety of empirical contexts is leading to a new and promising phase in the study of plant evolutionary radiations (Donoghue & Sanderson, 2015). Attention is shifting away from single traits acting at single points in a phylogeny, and toward the identification of sequences of evolutionary events or environmental circumstances that act in concert to alter speciation and/or extinction rates. This will help us to more accurately specify the causes of variation in diversification rates and to better explain the characteristic shapes that we see in the tree of life – not only radiations, but the existence and phylogenetic distribution of depauperate lineages as well.

Here we present analyses aimed at understanding diversification in *Viburnum* (Adoxaceae), a clade of *c.* 165 species of shrubs and small trees that are mainly distributed in temperate forests around the Northern Hemisphere. Today, the greatest species diversity is found in central and southern China, but the center of phylogenetic diversity is in Southeast Asia, where several early-diverging lineages occupy tropical forests (Clement & Donoghue, 2011; Clement *et al.*, 2014). Although *Viburnum* species exclusively inhabit mesic forest environments, their ecological tolerances vary widely, from species such as *V. edule* in the cold boreal forests of North America to *V. amplificatum* in the lowland tropical rainforests of Borneo.

Viburnum spans a phylogenetic scale that may have some distinct advantages. It is large enough to include repeated patterns and potentially to afford sufficient statistical power to identify causal factors, yet small enough that we can know its taxonomy, its characters and its ecology in considerable detail, and can achieve nearly complete species sampling in phylogenetic analyses. To date, patterns of diversification in *Viburnum* have been studied only by Moore & Donoghue (2007, 2009), based on a sample of just 52 species. They reported an increase in diversification rate for the *Regulaviburnum* clade (*sensu* Clement *et al.*, 2014), which includes all species except the Borneo endemic, *V. clemensiae*, and an additional increase in the *Oreinodentinus* clade, which includes montane Neotropical species. They argued that these radiations were driven not by trait evolution, but

instead by biogeographic movements. Our analyses tested these ideas and a variety of other diversification hypotheses with greatly expanded taxonomic coverage.

We are interested not just in the upward shifts in diversification rate, but in how best to account for scattered depauperate lineages (the 'depauperons' of Donoghue & Sanderson, 2015), especially the few rare species living today in lowland tropical forests. Therefore, we explore heterogeneity in speciation and extinction rates not just among subclades, but also associated with different biomes and with the shifting of climates through the Cenozoic. This leads us to develop what we will refer to as the 'dying embers' hypothesis, wherein speciation rates have decreased and extinction rates have increased toward the present in what we infer to have been the ancestral lowland tropical forest environment. As similar distribution patterns characterize other familiar northern temperate plant lineages, such as *Prunus* (Rosaceae) and *Acer* (Sapindaceae), we suppose that our findings on *Viburnum* diversification could apply quite broadly. Even more generally, we hope that our analyses will encourage increased attention to patterns of diversification associated with biome shifts of all sorts, and especially the possibility that there have been repeated pulses of diversification associated with such environmental transitions.

Materials and Methods

Taxon sampling

Here we present the most comprehensive analysis of *Viburnum* phylogeny to date, increasing the number of species analyzed based on 10 genes from 113 (Clement *et al.*, 2014) to 138. Of the 25 additional species marked by asterisks in Fig. 1, 17 were included in the barcoding analysis of Clement & Donoghue (2012), but based on a smaller number of markers; here we add numerous sequences for these species and for the first time include eight species from our recent collections in the Andes of Colombia. We are currently revising the taxonomy of the South American species of *Viburnum* (P. W. Sweeney *et al.*, unpublished), and have already reduced the number of accepted species from 35 (based on Killip & Smith, 1931; and several species described since then) to the 16 included here. We note, however, that this number includes samples from two localities in Ecuador cited by Vargas (2003), who proposed two new species ('*obtectum*' and '*stipitatum*') that were never formally published, and we anticipate further reductions (not increases) as our studies proceed. Several other *Viburnum* clades are in need of taxonomic attention, including *Succotinus* and *Solenotinus*, which figure importantly in the discussion later. These studies are well underway; in the meantime, we have here maintained most of the species in these groups recognized in Hara's (1983) treatment of *Viburnum* for Japan, Kern's (1951) treatment for Southeast Asia, and Yang & Malécot's (2011) treatment for China. Although we anticipate minor taxonomic adjustments in the near future, the taxonomy is robust enough now that such changes are highly unlikely to alter the basic results reported later.

DNA sequence matrix and phylogenetic analyses

We assembled a 10-gene data matrix (9772 aligned bp) for 138 species (TreeBASE: http://purl.org/phylo/treebase/phylows/study/TB2:S16844). This matrix included sequences from phylogenetic studies by Clement & Donoghue (2011), Chatelet *et al.* (2013) and Clement *et al.* (2014). We added four additional gene regions for 24 species first included by Clement & Donoghue (2012) and sequenced all regions for eight species (all from the *Oreinotinus* clade) that are new to *Viburnum* phylogeny (Supporting Information Table S1). The molecular regions sequenced included three chloroplast coding regions (*mat*K, *ndh*F, *rbc*L), six chloroplast noncoding regions (*pet*BD, *psb*A–*trn*H, *rpl*32–*trn*L, *trn*C–*ycf*6, *trn*K, *trn*S–*trn*G), and one nuclear region (internal transcribed spacer, ITS). The resulting matrix was over 87% complete; all species are represented by at least three genes. New sequencing for this project followed the protocols of Clement & Donoghue (2011), and all of the data are available in Genbank (Table S1).

Maximum-likelihood (ML) phylogenetic analyses of individual gene regions were conducted using RAxML (Stamatakis, 2006); these trees were inspected to identify any potential conflicts before conducting combined analyses. Preliminary combined analyses, also conducted in RAxML (Stamatakis, 2006), resolved most major clades recognized by Clement *et al.* (2014). There was little support, however, for the currently unnamed clade containing the *Opulus* clade and the *Laminotinus* clade (including *Coriacea*, *Sambucina*, *Succotinus* and '*Lobata*'), which was recovered with high bootstrap support (82%) and posterior probability (> 0.95) by Clement *et al.* (2014). This discrepancy is a function of the data analyzed. Here we analyzed only the 10-gene dataset, whereas Clement *et al.* (2014) analyzed a 129-gene dataset that included next-generation plastid sequence data from 22 *Viburnum* species. Working with the full dataset was not feasible here, so we instead constrained the monophyly of the *Opulus–Laminotinus* clade for further analyses.

Before analyzing the 10-gene combined dataset we tested various partitioning schemes. Using PartitionFinder v1.1.1 (Lanfear *et al.*, 2012), we compared three partitioning schemes and identified the best-fitting model of sequence evolution using the 'greedy' search scheme and limiting the models of evolution to those that can be implemented in MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist *et al.*, 2011). The three different partitioning schemes compared included: a single partition for all the data, one partition for all chloroplast regions and one for ITS, and one partition for the three chloroplast coding regions, one for the six chloroplast noncoding regions, and one for ITS. The three-partition scheme was favored using the Bayesian information criterion (BIC), and a GTR + I + G model of sequence evolution was used for all three partitions.

Trees were inferred in MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist *et al.*, 2011) with two independent runs, each with four chains and 30 million generations (sampled every 1000 generations) and with model parameters among partitions unlinked. *Viburnum clemensiae* was used to root the tree based on prior analyses using outgroups (Donoghue *et al.*, 2004;

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Fig. 1 The 165-tip maximum clade credibility tree for 138 *Viburnum* species based on 10 genes, showing the placement, based on morphological characters, of 27 species (shown in light gray) for which sequences are currently unavailable, with the root age fixed to 1. Species/sequences new to this study are marked with an asterisk (see Supporting Information Table S1). Posterior probabilities > 0.75 are indicated next to the branches, and those > 0.95 are indicated by thickened branches. Clade names are from Clement *et al.* (2014). Stars indicate the four nodes with the greatest evidence for a shift in diversification rate (see Fig. 2).

Winkworth & Donoghue, 2005; Clement & Donoghue, 2011; Clement *et al.*, 2014). Convergence and the adequacy of the burn-in period were both assessed using Tracer v1.5 (Rambaut & Drummond, 2009) (Supporting Information Fig. S1).

Dating analyses

The maximum clade credibility tree topology from MrBayes was fixed, whereas branch lengths were estimated in BEAST v1.7.5 (Drummond *et al.*, 2012) under a lognormal relaxed clock with a Yule prior for the distribution of node ages. The topology was fixed to facilitate comparisons across trees while still integrating over uncertainty in divergence time estimation. The crown age of *Viburnum* was fixed to one (uniform prior 0.99–1.01) and no internal nodes were constrained. Unlinked substitution models (GTR + I + G) were used for each partition. Two analyses were each run for 30 million generations, sampling every 3000 generations. Convergence and the adequacy of the burn-in were inspected visually in Tracer v1.5 (Rambaut & Drummond, 2009). Five hundred trees from the posterior distribution were selected randomly and used in subsequent diversification analyses to integrate over alternative sets of relative ages.

To infer absolute clade ages, we dated the *Viburnum* phylogeny using two different fossil calibrations (see Supporting Information Methods S1 for detailed justification). Each dating analysis was run for 10 million generations, sampling every 1000, and convergence and burn-in were again assessed with Tracer v1.5 (Rambaut & Drummond, 2009). The first analysis was calibrated using controversial leaf fossils from the Paleocene (e.g. Manchester, 2002), a few of which could belong to the *Porphyrotinus* clade within *Viburnum*. For this analysis, we used a lognormal prior on the ancestor of the maximum (stem) *Porphyrotinus* clade offset by 55 Myr, with an SD of 1. The root node was unconstrained, yet both median values and confidence intervals for the root of *Viburnum* were compatible with dates reported by Bell & Donoghue (2005). The second dating analysis instead used fossil pollen grains from two localities in the mid-Eocene for calibration (Gruas-Cavagnetto, 1978; McIntyre, 1991). Based on this pollen evidence, we placed a lognormal prior on the node subtending the *Valvatotinus* clade offset by 45 Myr, with an SD of 1. The root of *Viburnum* was again unconstrained, but also fell within the confidence intervals of Bell & Donoghue (2005).

Missing taxa

To achieve a comprehensive understanding of diversification in *Viburnum*, we carried out a number of analyses in which we added the 27 species that have not yet been sequenced to the 138-species trees (Fig. 1). These placements were based on morphological data obtained from recent regional taxonomic treatments (e.g. Yang & Malécot, 2011) and on our own extensive field, herbarium and anatomical studies (see Methods S2 for detailed justification). To more specifically situate each missing species, we randomly sampled a divergence time from a uniform distribution bounded by zero and the stem age of the sister taxon (see Methods S2, Fig. S2 for further details).

We further explored how taxon sampling affected the analysis of diversification rates by creating a series of subsampled *Viburnum* phylogenies corresponding to phylogenetic knowledge at different points in time. We therefore repeatedly pruned our maximum clade credibility tree with 138 species down to match the 41 species sampled by Winkworth & Donoghue (2005), the 90 species sampled by Clement & Donoghue (2011), and the 111 species sampled by Clement & Donoghue (2012). For the latter, we used 110 species, as *V. antioquiense* has since been synonymized with *V. tinoides*.

For each of these subsampled trees, the pruned species and the 27 missing species were assigned back to the appropriate tip remaining in the phylogeny so that all 165 species were accounted for in each iteration, but with differing degrees of phylogenetic resolution. In each case, placing the missing species back into the phylogeny required collapsing branches. The 41-tip tree, for instance, was missing species that occurred at deep nodes in the tree, and therefore, to incorporate all the missing taxa, the entire tree had to be reduced to 16 clades. The 90-species tree was reduced in this way to 45 tips, and the 110-species tree was reduced to 60 tips (see later Fig. 3).

Lineage diversification

We ran turboMEDUSA (http://www.webpages.uidaho.edu/~lukeh/software/software.html), an implementation of the MEDUSA function implemented in the R package GEIGER (Harmon *et al.*, 2008) following Alfaro *et al.* (2009). We used 500 BEAST posterior trees with 138 species (i.e. without the missing species) and the same 500 posterior trees with the missing taxa added so that each tree had 165 tips. Because the topology was constrained to remain constant in BEAST, the 138-species trees were identical except in branch lengths. The 165-species trees, however, differed in both branch lengths and topology, according to how the missing taxa were resolved. To summarize the results from the 165-species tree, we mapped all shifts onto the common 138-species tree topology; that is, for each shift, we identified the tip species in the derived rate class that were common to both trees, located the most recent common ancestor of those species in the 138-species tree, and placed the shift on the branch leading to that node. We used the default Akaike information criterion (AICc) threshold for both sets of trees (5.924318 for 165 tips and 5.514089 for 138 tips), but also explored alternative AICc cutoff values (Methods S3; Figs S3, S4).

We also evaluated diversification in *Viburnum* using Bayesian analysis of macroevolutionary mixtures (BAMM; Rabosky, 2014). The BAMM model fits a series of diversification processes to the tree using reversible jump Markov chain Monte Carlo to explore parameter space. We ran BAMM across 100 of the same 'complete' trees (with 165 tips) analyzed with turboMEDUSA. We used a restricted model that constrains each rate regime to be time-constant (analogous to turboMEDUSA) and set the priors for the analyses using the setBAMMpriors function in BAMMtools (Rabosky *et al.*, 2014). We used a poissonRatePrior of 1.0 and allowed shifts to occur on all branches

(minCladeSizeForShift = 1). BAMM was run for 10 million generations sampling every 10 000. Convergence and effective sample size were assessed in BAMMtools. We identified all shifts contained in the 95% credible set of shift configurations with a Bayes factor criterion of 10.

Character-dependent diversification analyses

To test for a correlation between certain character states and diversification, we used binary-state speciation and extinction (BiSSE) over a distribution of 500 'complete' trees (Maddison *et al.*, 2007; FitzJohn *et al.*, 2009). Each complete tree was generated by selecting a tree from the BEAST posterior distribution and adding the missing taxa to it using the method described earlier. We tested for an effect of tubular corollas, sterile marginal flowers, bud scales, leaf teeth, extrafloral nectaries (EFNs) and fruit color on each tree by comparing a four-parameter model with equal diversification rates (one speciation rate, one extinction rate, a transition rate from state 0 to state 1, and a transition rate from 1 to 0) to a six-parameter model where each character state had its own speciation and extinction rate (i.e. with one speciation rate and one extinction rate for each state). Character scorings were based on our own field and herbarium observations, and in several cases on species descriptions in Kern (1951) or Yang & Malécot (2011). In rare cases the character state for a species was either ambiguous or unknown and was scored as 'NA' (Fig. S5). Given that we are testing a number of characters with BiSSE, we were concerned that potentially high type I error rates might lead to spurious associations between individual characters and diversification. To test how BiSSE performed on our tree, we therefore conducted a limited series of tests based on simulated characters (see Methods S4 for details).

Ancestral biome reconstructions

We used the maximum clade credibility tree from both dating analyses (i.e. with the leaf fossils constraint and the pollen fossils constraint) to estimate transition rates and infer evolutionary shifts in biome occupation. To generate a distribution of 'complete' trees, we took the 138-species maximum credibility tree and added the missing taxa to it multiple times to obtain 500 trees each with the same underlying tree topology, but with a random assignment of the missing taxa. We used GEIGER (Harmon *et al.*, 2008) to estimate transition rates between lowland tropical and temperate environments on each tree, first constraining the model to symmetrical transition rates between states, and then allowing the rates to vary asymmetrically. Model fits were compared based on AICc scores. In analyses with asymmetry, the transition rates in each direction (from tropical to temperate and *vice versa*) were optimized in GEIGER under ML. To specifically test the tropical root hypothesis, we also compared models in which the root state was fixed to be either temperate or tropical and the transition rates were optimized in corHMM (Beaulieu *et al.*, 2013).

Diversification analyses with dated trees

Time-dependent BiSSE To test for variation in character-dependent diversification rates through time, we employed a version of BiSSE that allows different rate dynamics before and after a given time slice (Maddison *et al.*, 2007; FitzJohn, 2012). We used our two maximum clade credibility trees (dated with either leaf or pollen fossils) and again generated a distribution of 165-species trees by repeatedly adding the missing taxa to the 138-species maximum clade credibility trees. We fit BiSSE models before and after a time slice at 23 Myr ago (Ma) that corresponds to a period of rapid climate change and shifts in the global distribution of biomes at the end of the Oligocene.

Lineage through time (LTT) plots We constructed LTT plots based on 500 posterior distribution trees for each set of fossil dates (without the missing species). For each tree we separated the timing of temperate and lowland tropical diversification by assuming that the tropical state is ancestral and homologous across *Viburnum*. This assumption is justified by our transition rate estimations (see later).

Results

Lineage diversification

Figure 1 shows the maximum clade credibility tree from our Bayesian analysis of 138 *Viburnum* species based on 10 gene regions with the crown fixed to 1 (TreeBASE: study ID 16848; Dryad: doi: 10.5061/dryad.t5kf1). These results are consistent with analyses based on 113 species, and support all of the major clades recognized in the recent phylogenetic classification (Clement *et al.*, 2014). We note that the *Lobata* clade (including *V. acerifolium*, *V. orientale* and *V. kansuense*, all with trilobed leaves), which was recovered in earlier studies (e.g. Winkworth & Donoghue, 2005; Clement & Donoghue, 2011), does not appear here as a clade (in agreement with Clement *et al.*, 2014); branch support values are, however, low in this region of the tree.

TurboMEDUSA analyses using 500 trees from the posterior distribution of trees dated with the crown age set to 1 identified significant increases in diversification rate at 14 different nodes. Fig. 2 summarizes the results from the 165-species analysis (displayed on a 138-species tree; see earlier). This draws attention to three regions in the tree within which diversification shifts are likely to have occurred. Although several of these nodes were identified in close to half of the trees, branch length variation resulted occasionally in the selection of nearby nodes in regions surrounding the most commonly identified nodes. The number of diversification rates found per replicate tree for 500 trees is shown in Fig. 2(a) for the 138-species analysis and Fig. 2(b) for the 165-species analysis using the default AICc cutoff. Individual trees with 138 species or with 165 species mostly recovered three diversification rates (i.e. they identified two shifts in diversification rate), but 165-species trees recovered four rates (three rate shifts) about a quarter of the time (Fig. 2).
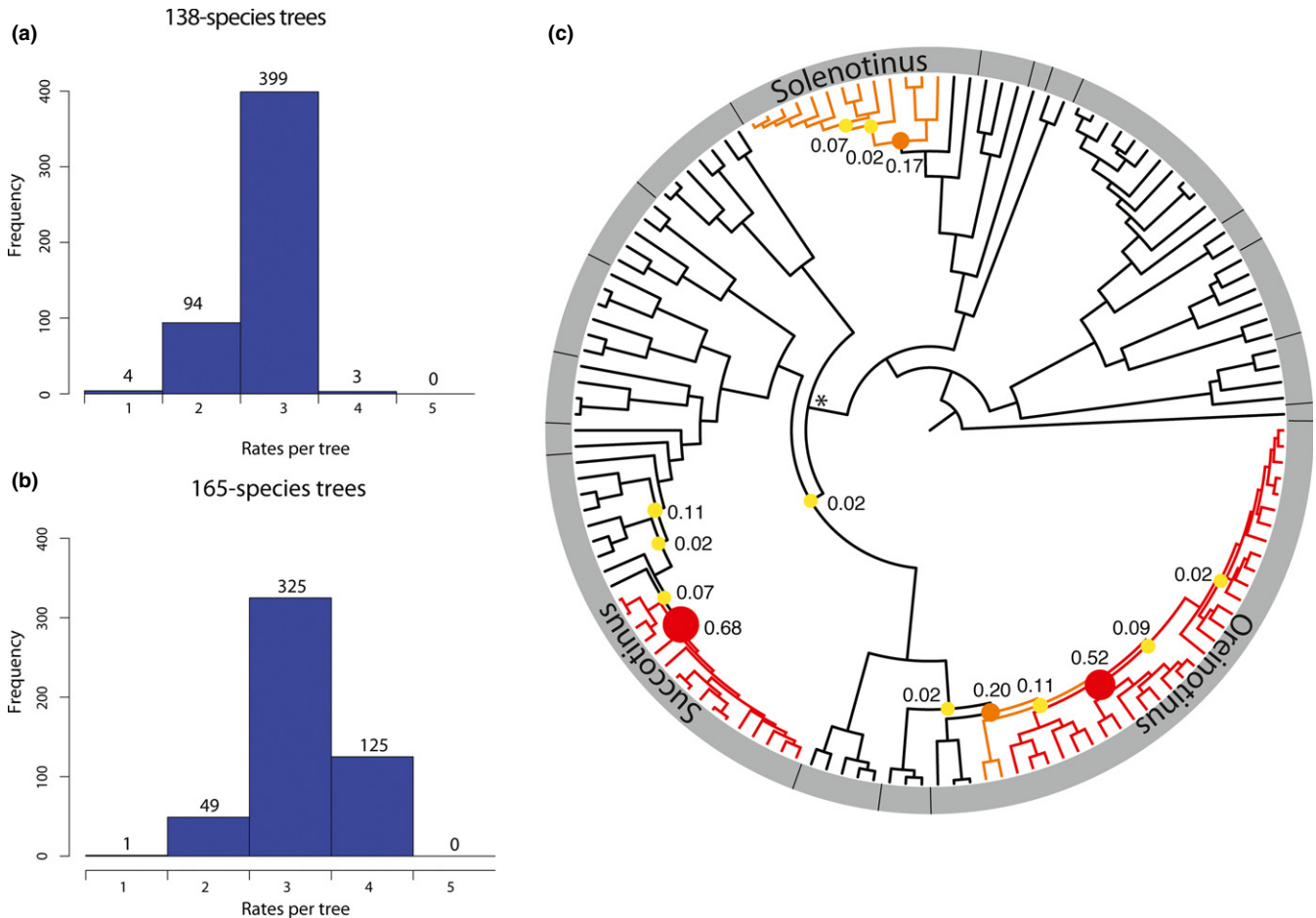
Fig. 2 (a) Histogram of the number of trees found to have 1–5 distinct diversification rates based on the 138-species trees. (b) Number of trees with 1–5 distinct diversification rates based on the 165-species trees. (c) TurboMEDUSA results for 500 trees with 165 species displayed on the 138-species tree. Dots indicate the placement of shifts identified by turboMEDUSA in at least 1% (five) of the trees; sizes of dots are proportional to the frequency of the shift and the proportion of trees with a shift in each location is indicated next to each dot. The asterisk marks the crown of *Nectarotinus*, the clade with EFNs. Black lines around the perimeter of the phylogeny demarcate the major clades shown in Fig. 1; clades containing significant shifts are labeled.

The three phylogenetic regions of interest correspond roughly to the *Oreinotinus* clade of the Neotropics, and to two clades of mainly temperate eastern Asia – a clade within *Succotinus* and another within *Solenotinus*. Support for a significant increase in diversification rate is especially strong for clades within *Oreinotinus* and *Succotinus*, and less so for *Solenotinus*. We rarely recovered a significant increase in a more inclusive clade that includes both *Oreinotinus* and *Succotinus* (the *Imbricotinus* clade of Clement *et al.*, 2014), and we very rarely identified (< 1% with the default AICc threshold, but see Fig. S4) a decrease in rate in the *V. clemensiae* lineage stemming from the root of the tree.

These results are sensitive to species sampling, as shown in Fig. 3. Using the 41-species dataset from Winkworth & Donoghue (2005), with the missing species added to the appropriate clades, we recovered three diversification rates (two rate shifts). With this highly reduced tree, there was one increase detected in the *Oreinotinus* clade, and a decrease detected in *V. clemensiae*. Both the 90-species dataset from Clement & Donoghue (2011) and the 110-species dataset from Clement & Donoghue (2012) also yielded three diversification rates (two shifts), with increases

placed in *Succotinus* and *Oreinotinus*. In these two more resolved trees, the placement of the *Oreinotinus* shift is more precise and matches the highest supported location of the shift in both our 138-species and 165-species trees. Overall, fewer diversification rates (and rate shifts) were recovered with smaller samples, despite the inclusion of all unsampled species in each case. The results obtained with the full dataset (Fig. 2) are achieved only as the sampling approaches that of our most comprehensive analyses.

BAMM analyses were generally concordant with our turbo-MEDUSA results, identifying the same three radiations at least occasionally (Fig. S6). In 33 out of 100 trees, the model with the highest posterior probability contained a single rate regime (no shifts). For 52 trees the best-supported model entailed a single rate shift, whereas for 15 trees the best model identified two or more shifts. In addition to the branches immediately surrounding the radiations, shifts were frequently identified either along the branch leading to *V. clemensiae* or at the base of *Regulaviburnum* (the clade including all species except *V. clemensiae*). Shifts were also occasionally recovered along the branch leading to
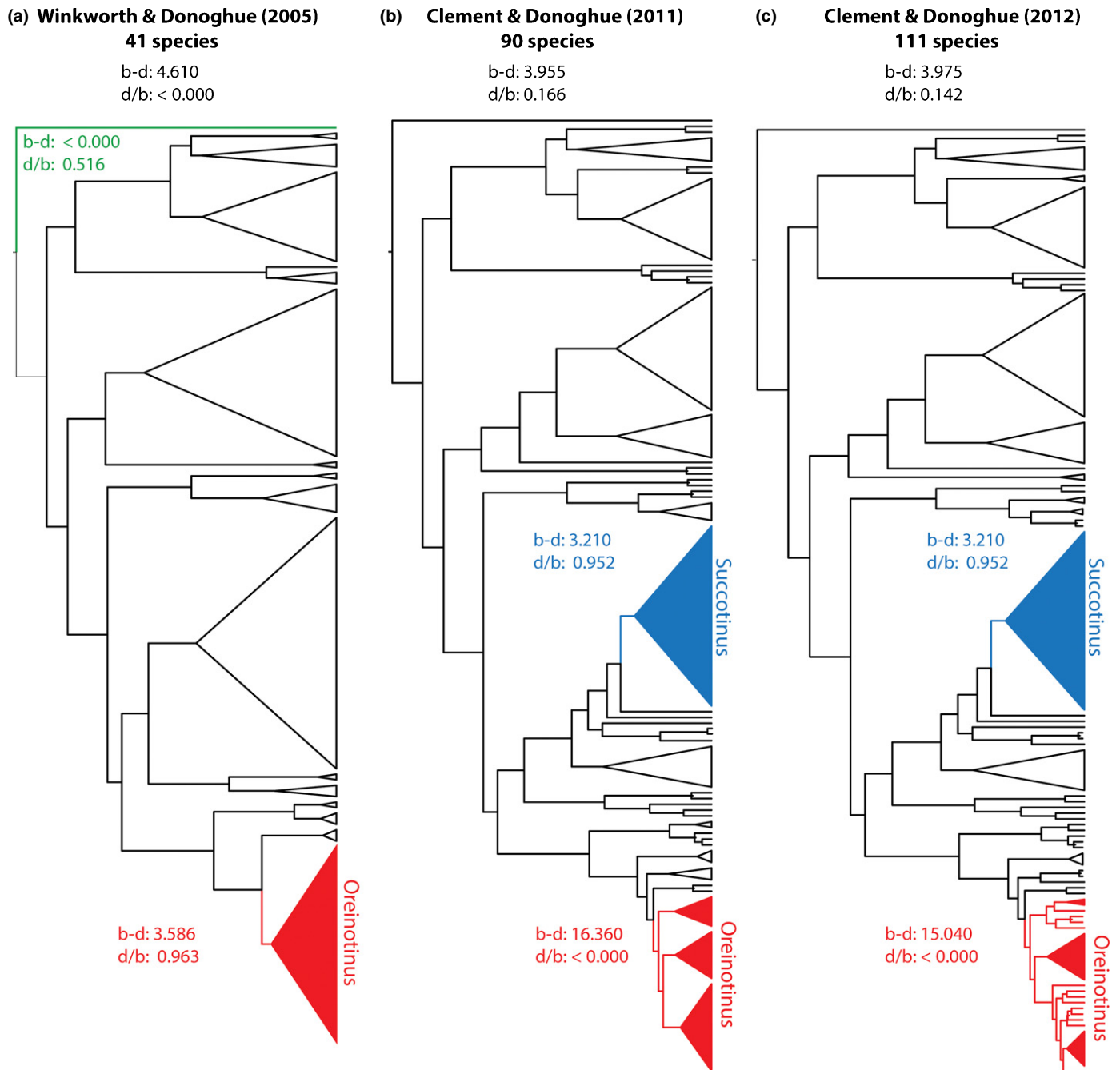
Fig. 3 TurboMEDUSA results based on three trees pruned to match the species sampling in (a) Winkworth & Donoghue (2005), (b) Clement & Donoghue (2011) and (c) Clement & Donoghue (2012). Shifts in diversification rate are identified by colored branches and rates associated with each regime are displayed. b, speciation rate; d, extinction rate.

Imbricotinus (Fig. S6). The fastest rates were typically in the *Orienotinus* and *Succotinus* clades, and these were up to twice as fast as the background rate.

Our BISSE analyses showed that several of the reproductive characters examined – sterile marginal flowers, tubular corollas and red fruits – are not associated with higher diversification rates (Fig. S7). However, purple fruits, which are characteristic of the *Porphyrotinus* and *Tinus* clades, were found to be significantly associated with diversification. We also found significantly higher diversification rates associated with EFNs, bud scales and leaf teeth.

EFNs evolved just once in *Viburnum* (Weber *et al.*, 2012; Clement *et al.*, 2014), along the branch indicated by an asterisk in Fig. 2, and are associated with a higher speciation rate in over 99% of the trees, and a decreased extinction rate in about half of the trees.

Ancestral state reconstructions

In order to explore differences in diversification associated with living in lowland tropical forests vs temperate forests (including cloud forests in the Neotropics and warm temperate forests

in Asia), we tested alternative models of character evolution and their associated ancestral reconstructions. Parsimony reconstructions favor a temperate root by three steps over the retention (homology) of an ancestral tropical lowland condition. When ML analyses are conducted, the more complex model with asymmetrical transition rates was significantly better in over 47% of the trees dated using both pollen and leaf fossils. In all other cases, the models were indistinguishable, with similar AICc scores (differing < 2); in no case was the symmetrical model significantly preferred. Under the constrained model, with equal rates of change between lowland tropical and temperate forests, a temperate root is preferred (probability over 0.89 in all trees), with 4–6 shifts later into lowland tropical forests (Fig. S8). However, when the constraint of symmetrical transition rates is lifted, the best model entails highly asymmetrical rates, with an average rate of 0.01725 into temperate regions and an average rate of 0.0002 into tropical regions. The near zero transition rate (≤ 0.001 for 98% of the trees) out of the temperate zone implies strict homology of the lowland tropical state; that is, with all modern species in lowland tropical forests having retained (conserved) the ancestral environment (Figs 4, S8). Under the fixed root models with asymmetrical rates, the lowland tropical root state was significantly better (AICc score difference ≥ 2) than a temperate root state in 94% of the trees. Ancestral state reconstructions using BiSSE on the pollen-dated tree likewise infer a lowland tropical root state with both 138 and 165 species (probability of a tropical root state > 0.90 in 79% of the trees). Using the tree dated with leaf fossils, BiSSE reconstructions prefer a temperate root and multiple shifts into the lowland tropics with the 138-species tree. By contrast, the same tree with the 27 missing taxa added (165 species) prefers the ancestral tropical reconstruction shown in Fig. 4 with the probability of a tropical root state ≥ 0.90 in 99% of the trees. In this case, the missing species (several of which are tropical) strongly affect the results.

Diversification analyses on dated trees

Figure 4 shows the results of our dating analysis using the 45 Myr old pollen fossils to calibrate the origin of the *Valvatotinus* clade. This centers the origin of crown *Viburnum* at *c.* 55 Ma, near the Paleocene–Eocene boundary, with the major subclades differentiating during the Eocene and into the Oligocene. With this calibration, the major radiations identified in *Oreinotinus*, *Succotinus* and *Solenotinus* took place in the mid- to late-Miocene, from 13 to 10 Ma. Within *Oreinotinus*, the movement into South America is centered on *c.* 5.5 Ma. The dated tree obtained using Paleocene fossil leaves to calibrate the origin of the *Porphyrotinus* clade is provided in Fig. S9. This analysis centers crown *Viburnum* on *c.* 80 Ma in the Campanian of the Late Cretaceous. Here the major *Viburnum* subclades extend back into the Paleocene and Eocene, and the significant radiations are in the earlier Miocene, between 16–20 Ma. In this case, movement into South America is centered on *c.* 8 Ma.

Figure 4 also shows the preferred ML reconstruction of the biome character (lowland tropical vs temperate), and the 23 Ma time slice used for the time-dependent BISSE approach designed to detect heterogeneity in diversification dynamics through time. Estimated net diversification rates for the lowland tropical and the temperate lineages before and after this time slice are shown in Fig. 5. With the time slice, the model finds that the lowland tropical lineages have experienced lower net diversification rates toward the present, whereas temperate lineages show higher diversification rates associated especially with the three identified radiations. Not surprisingly, this more complex model, with an extra six parameters, is preferred in only 1% of the 500 trees examined; however, we note that the two models have similar AICc scores (difference ≤ 2) in 91% of the trees.

LTT plots derived from the pollen-calibrated tree are shown in Fig. 5, where we contrast plots for all of *Viburnum* with plots for just the lowland tropical lineages and for just the temperate lineages. These show early origination and diversification of lowland tropical lineages, with a distinct leveling-off towards the present (Fig. 5b). By contrast, temperate lineages show a rise in origination later in the Eocene, with a sharp upward turn in lineage accumulation beginning in the mid-Miocene (Fig. 5c). Comparable LTT plots are provided in the Supporting Information for the leaf-calibrated time tree (Fig. S10). As expected, these show similar trends, but are set back further in time.

Discussion

Our study illustrates the value of comprehensive sampling for diversification studies. If we had carried out such analyses earlier, adding missing species to trees based on 41, 90 or even 110 species, we would not have identified all three of the regions with increased diversification rates that we find with 138 and 165 species. The generality of this result, however, needs to be more thoroughly tested using simulations and other empirical examples.

Nowhere in *Viburnum* is there anything that stands out as a major adaptive radiation in the classic sense (e.g. Hawaiian silverswords; Baldwin & Sanderson, 1998). Nevertheless, we believe that the turboMEDUSA and BAMM analyses are correctly identifying diversification shifts in the *Oreinotinus*, *Succotinus* and *Solenotinus* clades, which, although unimpressive in terms of generating disparate morphological forms, have nevertheless rapidly generated multiple species (independently evolving lineages). In general, and in agreement with Moore & Donoghue (2007, 2009), potentially relevant morphological attributes are not directly connected to diversification shifts in *Viburnum*. Specifically, it appears that the several lineages in which sterile marginal flowers evolved, or in which tubular corollas originated, are not diversifying at an elevated rate (Figs S5, S7). Likewise, those with red juicy fruits are not diversifying significantly faster. The only reproductive character we tested that is potentially linked to elevated diversification is the near-simultaneous ontogenetic transition from green to pulpy purple fruits (found in *Oreinotinus*, which did radiate, but also in the *Tinus* and *Mollotinus*, clades which did not) (Figs S5, S7). Although lineages with several vegetative characters do show higher diversification rates (extrafloral nectaries (EFNs), bud scales, toothed leaves; Figs S5, S7), none of these appears to have directly driven these radiations (see later).
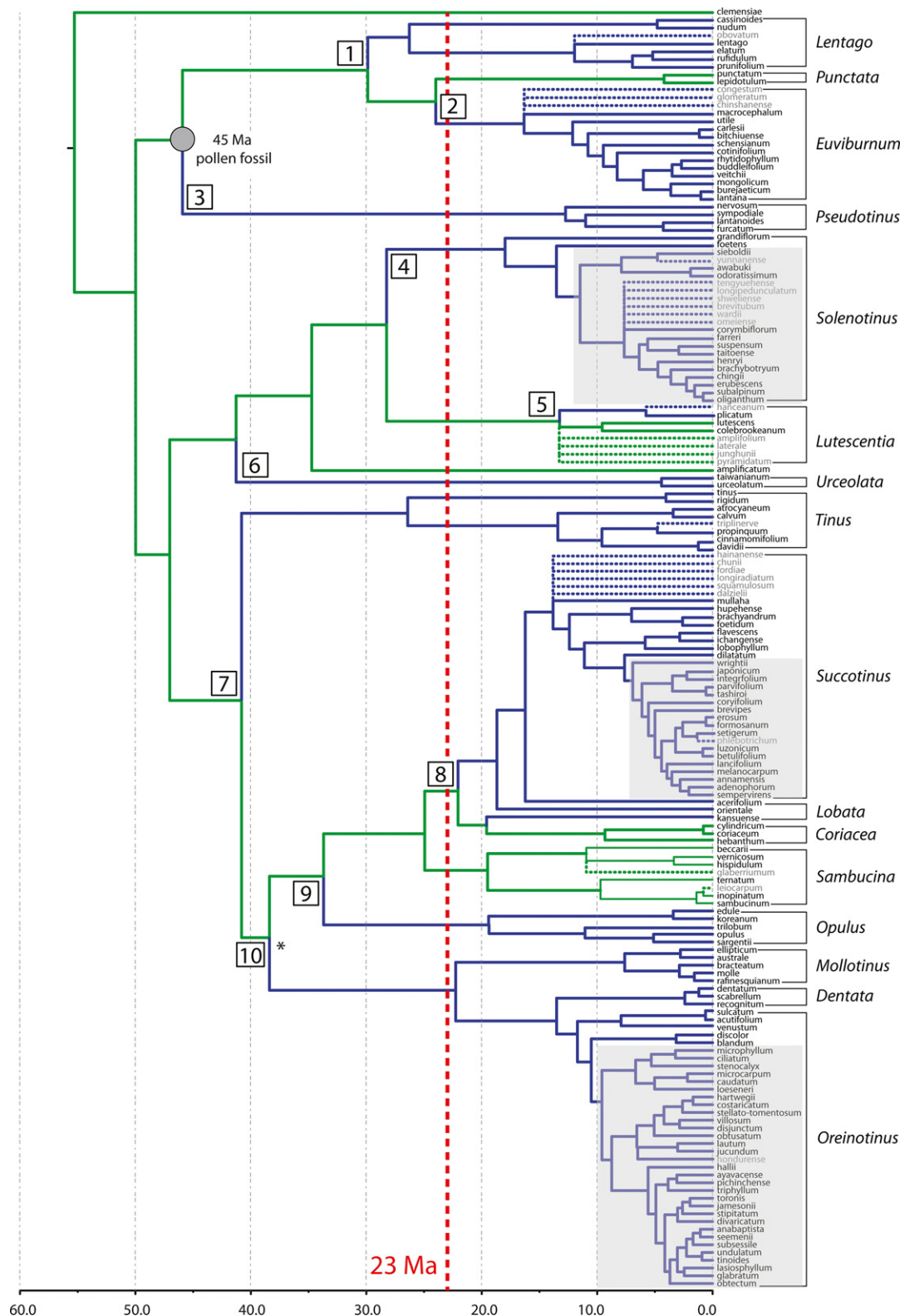
Fig. 4 The 138-species time tree calibrated with fossil pollen. The 27 added taxa are shown as polytomies with dotted lines. Branch colors show the maximum-likelihood (ML) character reconstruction with asymmetrical transition rates (green lines, lowland tropical; blue lines, temperate or cloud forest). Ten shifts to the temperate state are numbered; an additional shift, involving *Viburnum kansuense*, is not numbered as it is not confidently resolved. Gray boxes indicate regions of the tree identified as having significantly higher rates of diversification in turboMEDUSA analyses (see Fig. 2). The gray circle at top left marks the location of the fossil pollen calibration; an asterisk marks the node where the leaf fossils constraint is placed in the alternative dating analysis shown in Supporting Information Fig. S9. A red dotted line indicates the position of the 23 Myr ago (Ma) time slice tested with time-dependent binary state speciation and extinction (BiSSE) models (see Fig. 5a).
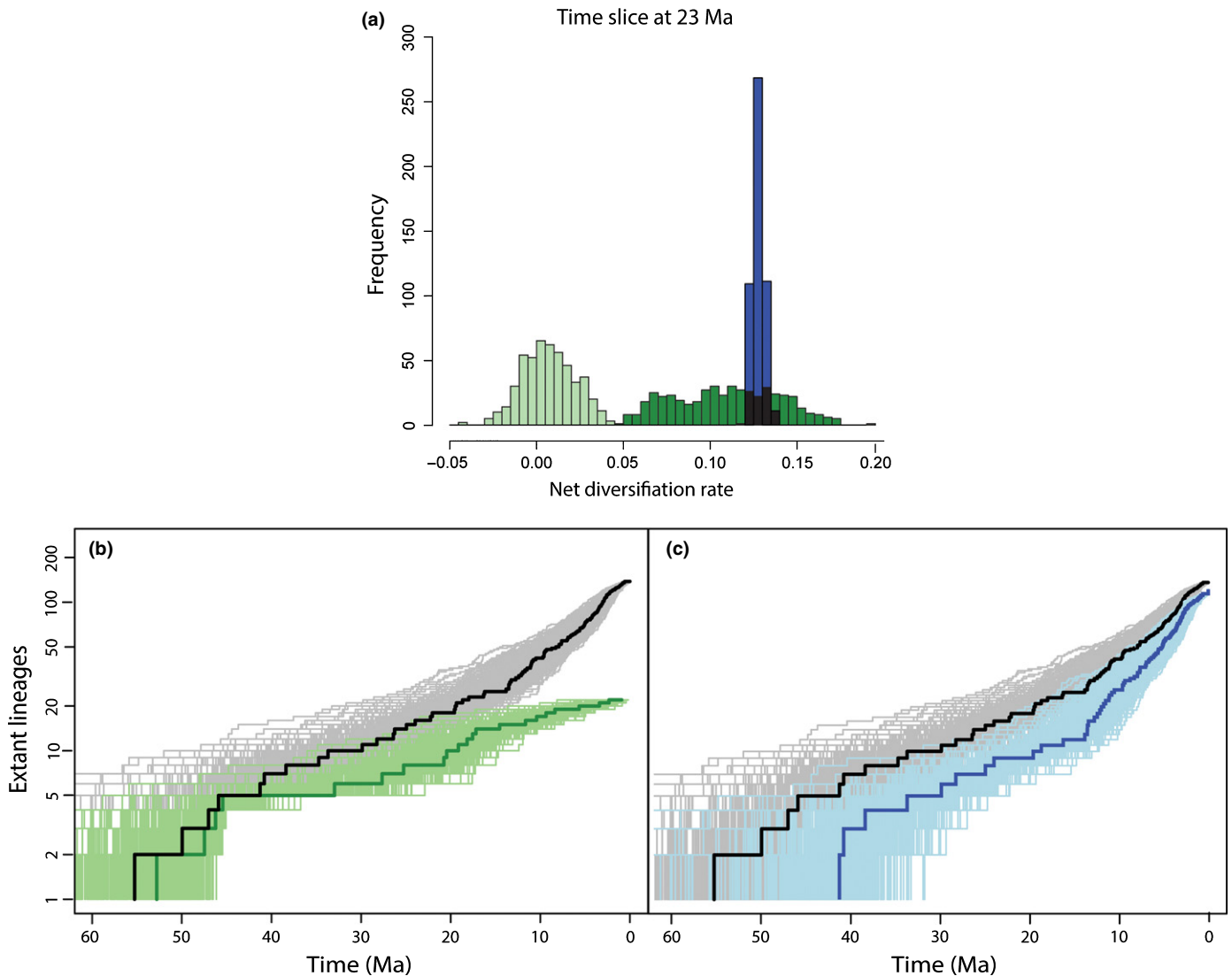
Fig. 5 (a) The distribution of net diversification rates found in 500 trees with different placements of missing taxa. Dark green bars, tropical diversification rates before the time slice (at 23 Myr ago, Ma); light green bars, tropical rates after the time slice; blue bars, temperate diversification rates after the time slice. (b) Lineage through time (LTT) plot based on the fossil pollen calibration. The black line indicates the log (number of lineages) over time for all *Viburnum* based on the maximum clade credibility BEAST tree. Gray lines show results for 500 posterior distribution trees. Dark green line, LTT for tropical lineages in the maximum clade credibility tree; light green lines, results for tropical lineages from 500 posterior trees. (c) Dark blue line, LTT for temperate lineages in the maximum clade credibility tree; light blue lines, results for temperate lineages for 500 posterior trees. Gray and black lines as in (b).

Our binary state speciation and extinction (BiSSE) analyses do show that EFNs are linked with higher diversification, which deserves special attention because EFNs have been identified as a key innovation in other clades. *Senna* (Fabaceae) is the most thoroughly explored case (Marazzi & Sanderson, 2010), but a correlation with diversification has also been noted in other lineages, including *Viburnum*, by Weber & Agrawal (2014). The mechanism connecting EFNs to enhanced diversification is unclear. It has been argued that EFNs serve to increase individual fitness, and that this might translate into larger population sizes, larger geographic ranges and/or the occupation of a wider variety of habitats (Marazzi & Sanderson, 2010; Weber & Agrawal, 2014). These factors might increase the likelihood of speciation, but could be more directly linked to a decreased rate of extinction. If

so, EFNs could be viewed as enhancing the persistence of species and whole clades, which might in turn increase the likelihood of experiencing later evolutionary events that increase speciation (e.g. radiation into the Andes in the case of *Senna*). This 'indirect innovation' explanation is favored by Weber & Agrawal (2014). Alternatively, it is possible that EFNs are associated with increased diversification in BISSE analyses simply because they evolved in the ancestor of a clade that includes two of the three major radiations in *Viburnum* (*Oreinotinus* and *Succotinus*). That is, this could be a case of a trait that has 'hitchhiked' to higher frequency owing to subsequent radiations, as opposed to being a causal agent.

In our analyses, bud scales and leaf teeth might also be interpreted as hitchhikers, as it is difficult to imagine a direct causal

connection between these traits and either speciation or extinction beyond their influence on individual fitness (their adaptive value). These are most likely simply correlated with shifting into cold, highly seasonal climates, which also happen to be the climates within which the three major *Viburnum* radiations occurred. Likewise green-to-purple fruits may be only incidentally correlated by virtue of their presence in the highly significant *Oreinotinus* radiation.

If *Viburnum* radiations have not been driven by key morphological innovations, were they triggered instead by key opportunities, as Moore & Donoghue (2007, 2009) suggested? This seems a likely explanation especially for *Oreinotinus*, which radiated in cloud forests throughout the mountains of Mexico and Central America and into the Andes between 5 and 10 Ma. Here the likely explanation would appear to be rapid allopatric speciation in a topographically heterogenous area that promotes isolation-by-distance (see Fjeldså *et al.*, 2012; and, for the Andes in particular, McGuire *et al.*, 2014; Luebert & Weigend, 2014). This fits with the narrow and disjunct geographic ranges of most of the Neotropical species, with the relatively minor morphological differences that sometimes distinguish closely related species in this region, and with the limited extent of sympatry and the extensive hybridization that sometimes ensues on secondary contact (Donoghue, 1982). We suspect that the putative radiation within *Solenotinus* had a similar cause, as many of the most recently formed species in this clade are native to the eastern Himalayan region in China.

It is less clear that a simple geographic heterogeneity argument applies to the radiation in *Succotinus*, which is widely dispersed across eastern and Southeast Asia at many different elevations. One possibility in this case is that multiple evolutionary shifts, perhaps in both directions, between colder deciduous temperate forests and warmer evergreen temperate forests, have driven speciation. These shifts have repeatedly been associated with changes in characters that distinguish closely related species, especially entire vs toothed leaf margins. Specifically, entire-margined, evergreen leaves appear to have evolved repeatedly; for example, in *V. integrifolium*, *V. hainanense* and *V. sempervirens*. If this is the case, then there would be an interesting contrast. In *Oreinotinus* and *Solenotinus*, environmental niches seem to have been quite conserved, and niche conservatism has fostered successive isolation, along the lines of the model developed by Wiens and colleagues (Wiens, 2004; Kozak & Wiens, 2006; Hua & Wiens, 2013). By contrast, in *Succotinus* it is possible that frequent niche shifts have been responsible for diversification.

Turning to the issue of the ancestral environment and possible connections between diversification and evolutionary biome shifts, we emphasize first the difficulty of confidently making such assessments when only 22 living *Viburnum* species are scored as occupying lowland tropical forest environments. Under standard parsimony it is more costly (three steps) to reconstruct the ancestor of *Viburnum* as living in topical forests. ML analyses favor a temperate root when transition rates are forced to be equal between the two states, but infer a lowland tropical root when transition rates are allowed to differ in the two directions. We favor the results based on allowing asymmetrical transition rates.

We know of no evidence supporting symmetrical transition rates; on the contrary, there is strong evidence from multiple studies that biome shifts tend to occur asymmetrically (Donoghue & Edwards, 2014). And, in plants, at least, transition rates appear to have been much higher out of tropical forests into temperate forests than in the reverse direction (Judd *et al.*, 1994; Donoghue & Edwards, 2014; Kerkhoff *et al.*, 2014). When we tested alternative root states for *Viburnum* using an asymmetrical model, the tropical root was found to be significantly better. BiSSE results for this character depend on the species sampling, but the lowland tropical root was preferred with more complete coverage.

In our view, the best interpretation at this stage is the one based on allowing transition rates to differ in the two directions, namely that the lowland tropical forest environment is ancestral for *Viburnum*. In spite of the mixed statistical support for this scenario, several independent lines of evidence also argue for a 'tropical to temperate' trajectory. We are especially struck by the observation that many temperate *Viburnum* species are characterized by seed germination requiring 'double dormancy' (or, more specifically, deep simple epicotyl morphophysiological dormancy: Baskin *et al.*, 2008; Chien *et al.*, 2011), in which alternating warm and cold (generally freezing) conditions are required for proper sequential development of the radicle and epicotyl. Dispersal of such seeds from colder northern forests into tropical latitudes would be successful only at higher elevations where there would be a chance of experiencing sufficiently cold temperatures. Yet, in Southeast Asia, the *Viburnum* species that we have scored as tropical are largely found at lower elevations where such temperatures would be exceedingly rare. The unlikely germination of seeds of temperate *Viburnum* species in lowland tropical environments tends to favor the idea that the movement was in the opposite direction. In general, in such complicated real cases we believe that such ancillary arguments – in combination with strictly phylogenetic arguments – will be necessary to tip the balance in favor of one explanation over another.

Working, then, on the assumption of lowland tropical ancestry, it appears that the three significant radiations in *Viburnum* took place in clades that shifted into temperate deciduous forests (or later into high-elevation cloud forests at more tropical latitudes in *Oreinotinus*), which experience cold temperatures on some regular basis. However, it is worth noting that there have been, according to our preferred ML reconstructions, at least 10 separate shifts within *Viburnum* from lowland tropical into temperate and cloud forests. This is consistent with the view that biome shifts, which have been viewed as relatively rare events (Donoghue, 2008; Crisp *et al.*, 2009), are, on closer inspection, probably more common but also tend to be clustered within particular clades (Edwards & Donoghue, 2013; Donoghue & Edwards, 2014). Of the 10 shifts in *Viburnum*, three were associated with later radiations, but the other seven were not and are relatively small clades (*Pseudotinus*, with four extant species; *Opulus*, five; *Urceolata*, two; *Tomentosa*, two; *Lentago*, seven; *Tinus*, eight; *Euviburnum*, 15). Thus, although the three radiations that we have identified took place in temperate clades, shifting into the temperate forest biome has not, by itself, consistently resulted in radiation. Instead, these radiations presumably

depended more directly on the additional factors noted earlier. Under this view, shifts into temperate forests promoted movements into mountainous regions – even into mountains at tropical latitudes – which then resulted in increased speciation. It is this particular confluence of events that made the difference (cf. Donoghue & Sanderson, 2015).

This line of reasoning invites attention to the other side of the story, namely the species-poor lowland tropical *Viburnum* lineages. By our estimate there are only 22 truly lowland tropical species alive today, and, with the exception of the *Sambucina* clade, with eight species, and the *Lutescentia* clade, with seven tropical species, the remainders are depauperate and deep branches within the tree. This applies especially to *V. clemensiae*, the single species from Borneo that appears to be the sister of the remainder of *Viburnum*, but also to *V. amplificatum*, likewise from Borneo, which branches early in the *Amplicrenotinus* clade, and *V. punctatum* and *V. lepidotulum* from Southeast Asia, an early-branching lineage within *Valvatotinus*. Why are these lowland tropical lineages not more diverse? Were they ever diverse in the past? Is it possible that different dynamics have governed the diversification of the lowland tropical lineages vs the temperate and tropical montane lineages?

The analyses presented in Figs 4 and 5 support the idea that the dynamics have indeed differed over time. Taken at face value, they suggest that *Viburnum* originated and diversified initially in the Eocene in tropical forests in Southeast Asia. Later there were at least 10 shifts out of these forests into temperate forests (broadly speaking), probably in the Old World, accompanied by a repeated set of morphological changes (e.g. toothed leaf margins, broadly ovate leaves; Schmerler *et al.*, 2012). Depending upon other factors, including the occupation of mountainous areas, three of these temperate lineages radiated to varying degrees in the Miocene. Following an early phase of diversification in the lowland tropics, diversification in the tropical lineages seems to have tapered off dramatically after the spread of cold climates in the Oligocene (Fig. 5). There have been a few recent speciation events in the lowland tropics, especially in the *Sambucina* clade, within which, for example, two Bornean species, *V. hispidulum* and *V. vernicosum*, diverged not long ago, but these are exceptions to the general pattern. The lineage through time plots in Fig. 5 also give the impression of a diversification slow-down in the lowland tropics as compared with a speed-up in at least some of the temperate lineages that caused the notable upward shift in the Miocene, a period of expanded colder (and drier) climates worldwide that coincided with multiple plant radiations (e.g. Arakaki *et al.*, 2011; Spriggs *et al.*, 2014). Our time-dependent BiSSE analysis also supports a model that entails less speciation and more extinction in lowland tropical lineages from the Oligocene onward, and, conversely, increased diversification in temperate lineages. Taken together these analyses imply that *Viburnum* was once more diverse in the lowland tropics of Southeast Asia, and has been slowly dying off in its ancestral home. Under this interpretation, the remaining lowland tropical lineages are 'dying embers' of a past era of tropical diversification.

One obvious problem with this interpretation is that lineages most directly related to *Viburnum* occupy temperate forests, not the lowland tropics. Nevertheless, it is at least plausible that the Dipsacales as a whole originated in more tropical environments. Dipsacales and Paracryphiaceae form the Dipsidae within a more inclusive Dipsapiidae with the Apiales (Tank & Donoghue, 2010). Parcryphiaceae (*Paracryphia*, *Quintinia* and *Sphenostemon*) are evergreen trees and shrubs variously inhabiting tropical rainforests mainly in New Caledonia, New Guinea, and Queensland, Australia. Early branching Apiales include *Pennantia*, Torricelliaceae and Pittosporaceae, which also are mainly tropical forest plants with Austral distributions. Within Adoxaceae, *Viburnum* is sister to *Sambucus* and *Adoxa* and its relatives. Although most of these species occupy cold environments around the northern hemisphere, it is noteworthy that several early-branching species of *Sambucus* (*S. australasica* and *S. gaudichaudiana*) grow in sub-tropical to tropical forests in eastern Australia. If Dipsacales and Adoxaceae did first occupy tropical forests, early lineages must have gone extinct, consistent with the theory advanced here.

Concluding thoughts

A key implication of the dying embers hypothesis that we have just outlined is that *Viburnum* is doing poorly in the environments in which it first evolved, and thriving in the environments into which it moved at a later date. Why would this be? We are not sure, but we think it is possible that the very attributes that predisposed *Viburnum* species to adapt multiple times to cold, seasonal climates, might somehow put them at a disadvantage in current lowland tropical environments. Is it possible that they are simply being outcompeted in their ancestral environment as superior tropical plants have diversified around them? Lowland tropical environments are, after all, exceedingly dynamic, and have undergone immense changes, including the development of new species interactions and the rapid radiation of many tropical lineages (e.g. *Inga*; Richardson *et al.*, 2001). In view of such changes, it is important to also consider that *Viburnum* might initially have radiated in a forest type that has no modern analogue.

In closing we note that the course of events that we have just hypothesized for *Viburnum* matches in some ways the 'taxon pulse' hypothesis that Erwin (1985) developed to explain macroevolutionary patterns in carabid beetles. His idea was that these beetles started out in aseasonal warm tropical environments and moved from there, multiple times, into increasingly harsh environments at higher latitudes and altitudes. Moreover, according to Erwin's theory, old lineages tended to go extinct in the ancestral environments, such that the surviving members are today found only in more extreme and evolutionarily derived habitats. If this scenario applies to *Viburnum* it might help to explain another curious aspect of the phylogeny, namely the fact that several of the clades that today are found only in the coldest climates actually branch deep within the phylogeny. This applies especially to the *Pseudotinus* and *Opulus* clades, both of which today include only a few species living mainly in boreal forests, and both of which are at the tips of exceptionally old *Viburnum* lineages. Is it possible that these represent clades that are near the end of their 'taxon pulse' and, if so, that tropical and temperate relatives have gone extinct along these lines?

It is important to note that such a scenario is especially problematical for phylogenetic inference of past distributions, as evolution is essentially 'covering its tracks' and leading us to infer pervasive niche conservatism when the reverse could be true. In the face of such concerted extinction, will we have the power to sort this out with any certainty? At the very least, this idea highlights the critical need to include as many as possible of the rare tropical species in phylogenetic studies of these mainly temperate clades. There is no reason to assume that *Viburnum* is a unique case, and we imagine that other key elements of temperate forests, such as *Acer* and *Prunus*, may have their own dying embers in tropical environments. We should at least entertain the possibility that many of our large temperate 'groups' are not groups at all, but rather are polyphyletic clusters of lineages derived via parallel biome shifts out of the tropics, masked by extinction.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Maximum clade credibility tree from MrBayes analysis.

Fig. S2 Example 165-species tree with the unsequenced species added.

Fig. S3 TurboMEDUSA AICc threshold simulation results.

Fig. S4 TurboMEDUSA results for the 138-species tree and alternative AICc threshold cutoffs.

Fig. S5 Characters used in binary state speciation and extinction (BiSSE) analyses.

Fig. S6 BAMM results for 100 complete trees.

Fig. S7 Binary state speciation and extinction (BiSSE) results for a set of morphological characters.

Fig. S8 Maximum-likelihood ancestral state reconstructions based on the transition rates estimated with GEIGER.

Fig. S9 Species tree (138 species) dated using fossil leaves from the Paleocene.

Fig. S10 Histograms and lineage through time (LTT) plots showing the distribution of diversification rates found based on trees dated using leaf fossils.

Table S1 Voucher information and GenBank accession information for all species included in this study

Methods S1 Fossil calibrations.

Methods S2 Placement of unsequenced species.

Methods S3 TurboMEDUSA AICc threshold tests.

Methods S4 Binary state speciation and extinction (BiSSE) character simulations.

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