

## Force of habit: shrubs, trees and contingent evolution of wood anatomical diversity using *Croton* (Euphorbiaceae) as a model system

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• **Background and Aims** Wood is a major innovation of land plants, and is usually a central component of the body plan for two major plant habits: shrubs and trees. Wood anatomical syndromes vary between shrubs and trees, but no prior work has explicitly evaluated the contingent evolution of wood anatomical diversity in the context of these plant habits.

• **Methods** Phylogenetic comparative methods were used to test for contingent evolution of habit, habitat and wood anatomy in the mega-diverse genus *Croton* (Euphorbiaceae), across the largest and most complete molecular phylogeny of the genus to date.

• **Key Results** Plant habit and habitat are highly correlated, but most wood anatomical features correlate more strongly with habit. The ancestral *Croton* was reconstructed as a tree, the wood of which is inferred to have absent or indistinct growth rings, confluent-like axial parenchyma, procumbent ray cells and disjunctive ray parenchyma cell walls. The taxa sampled showed multiple independent origins of the shrub habit in *Croton*, and this habit shift is contingent on several wood anatomical features (e.g. similar vessel-ray pits, thick fibre walls, perforated ray cells). The only wood anatomical trait correlated with habitat and not habit was the presence of helical thickenings in the vessel elements of mesic *Croton*.

• **Conclusions** Plant functional traits, individually or in suites, are responses to multiple and often confounding contexts in evolution. By establishing an explicit contingent evolutionary framework, the interplay between habit, habitat and wood anatomical diversity was dissected in the genus *Croton*. Both habit and habitat influence the evolution of wood anatomical characters, and conversely, the wood anatomy of lineages can affect shifts in plant habit and habitat. This study hypothesizes novel putatively functional trait associations in woody plant structure that could be further tested in a variety of other taxa.

**Key words:** Character state reconstruction, contingent evolution, *Croton*, Euphorbiaceae, functional traits, habit, habitat, phylogenetic comparative methods, wood anatomy.

### INTRODUCTION

Habit in woody plants – lianas, shrubs and trees – is typically an intrinsic trait (Cronquist, 1968; Sussex and Kerk, 2001; Barthélémy and Caraglio, 2007), and associated functional, structural and anatomical traits of wood are varying related to plant habit and also to habitat (Rowe and Speck, 2005; Martínez-Cabrera *et al.*, 2009; Willis and McElwain, 2013; Morris *et al.*, 2016). Broad surveys of wood anatomical diversity, across either disparate taxa or disparate geographies, can inform our understanding of the evolution of wood structure in the context of habit and habitat (van der Graff and Baas, 1974; Carlquist, 1975, 1977, 1980, 1985a, b; Carlquist and Hoekman, 1985; Baas and Schweingruber, 1987; Lindorf, 1994; Noshiro and Baas, 1998; Rosell *et al.*, 2007). Taking an explicitly phylogenetic approach to study the evolutionary interplay between wood structure and habit is also informative (Zanne *et al.*, 2010; Kattge *et al.*, 2011; Martínez-Cabrera *et al.*, 2011; Schweingruber *et al.*, 2014). Regardless of the influence of

ecology on wood evolution, wood anatomical traits (*sensu* Violle *et al.*, 2007; Beeckman, 2016) have long been used in plant systematics (Bailey and Tupper, 1918; Metcalfe and Chalk, 1950; Baas, 1982; Herendeen, 1996; Baas *et al.*, 2000; Herendeen and Miller, 2000; Carlquist, 2001; Olson, 2005, 2012), even as data for phylogenetic analyses (e.g. Loconte and Stevenson, 1991; Hufford, 1992; Nandi *et al.*, 1998; Kårehed, 2001; Luna and Ochoterana, 2004; Lens *et al.*, 2007). Today, studies using molecular phylogenies to track wood evolution have become increasingly common (e.g. Lens *et al.*, 2008a, b; Boura *et al.*, 2011; Appelhans *et al.*, 2012; Pace and Angyalossy, 2013; Schweingruber *et al.*, 2014; Pace *et al.*, 2015; Trueba *et al.*, 2015; Kidner *et al.*, 2016), as are the use of comparative methods to test wood evolutionary hypotheses and uncover patterns suggestive of functional relationships (Wiedenhoef, 2008; Zanne *et al.*, 2010, 2014; Martínez-Cabrera *et al.*, 2011; Olson and Rosell, 2013).

Modern phylogenetic comparative methods (PCMs) are capable of much more than statistical control for phylogeny

(Garamszegi, 2014) and can be used to model evolutionary process along phylogenies, such as speciation and extinction, the influence of traits on diversification, and trait evolution (see review in Pennell and Harmon, 2013). Nevertheless, the statistical non-independence among species trait values because of their relatedness remains an essential concept in these types of studies (Felsenstein, 1985; Freckleton *et al.*, 2002; Revell *et al.*, 2008). This non-independence can be measured by the phylogenetic signal (PS), or the ‘tendency for closely related species to resemble each other more than expected by chance’ (Blomberg and Garland, 2002, p. 905; Blomberg *et al.*, 2003). Traits exhibiting PS have probably evolved gradually through time (approximating a Brownian motion model of evolution) and thus we expect the phylogeny to be helpful in explaining the distribution of the character states among the taxa (Blomberg and Garland, 2002; Kamilar and Cooper, 2013). Traits with no PS may either be extremely labile or conversely extremely stable (Revell *et al.*, 2008; Blomberg *et al.*, 2003). Examining patterns of PS can provide information about evolutionary process and is considered a key prerequisite for studies of character evolution (Freckleton *et al.*, 2002) and their correlation with other traits (Kamilar and Cooper, 2013). Associations between two traits in multiple, distinct lineages provide strong evidence for the significance of the correlation (Maddison and FitzJohn, 2015). Additionally, specific tests for correlated evolution (Pagel, 1999) can reveal what features change in association with other characters, life-form characteristics (e.g. habit), or aspects of the environment (e.g. habitat). Although these tests do not inform us about the timing or relation between traits over the course of evolution, supplementary PCMs can be used to determine whether pairs of correlated traits exhibit contingent patterns of evolution (*sensu* Pagel, 1994).

To evaluate the contingent evolution of wood anatomical diversity in the context of plant habit, we chose one of the mega-diverse genera of flowering plants, *Croton* (Euphorbiaceae). With 1300+ species (Frodin, 2004), and a primarily pantropical and subtropical distribution, species of *Croton* can be found in both arid (xeric) and moist (mesic) habitats throughout its range (Webster, 1993). Growth habit in *Croton* includes herbaceous non-woody annuals, but most species are persistently woody shrubs and small to large trees. Molecular phylogenetic studies have helped circumscribe the genus and evaluate the monophyly and relationships of previously proposed taxonomic sections (Berry *et al.*, 2005a, b; van Ee and Berry, 2009, 2010; Riina *et al.*, 2009, 2010; van Ee *et al.*, 2006, 2008, 2011; Caruzo *et al.*, 2011). Wood anatomical studies in *Croton* have largely been restricted to descriptive work (Loureiro, 1968; Hayden and Hayden, 1994; Luchi, 2004), new species descriptions (Berry *et al.*, 2005a for *Brasiliocroton*; Riina *et al.*, 2010) or detailed treatments of groups of species (Pollito, 2004; Mennega, 2005), as well as studies of laticifers in the wood of several species (Rudall, 1987, 1989, 1994; Wiedenhoef *et al.*, 2009). Wiedenhoef (2008) was the first study to tackle the evolution of wood anatomy in *Croton* in an explicitly phylogenetic context, using parsimony-based phylogenetic comparative methods.

This study is the first report using PCMs to investigate the evolution of wood anatomical diversity in the context of habit and habitat in a mega-diverse genus. In this paper we restrict

our analysis to discrete, binary characters exhibiting phylogenetic signal, tested across the largest and most complete molecular phylogeny of the genus to date. By taking this approach in this group, we are able to provide an explicit contingent evolutionary framework showing that habit and habitat influence the wood anatomy of *Croton* and, conversely, the wood anatomy of lineages influences shifts in plant habit and habitat in ways not previously reported. Our work hypothesizes novel putatively functional traits in woody plant structure and calls for testing these hypotheses in other taxa.

## MATERIALS AND METHODS

### *Phylogenetic inference and node-age estimation*

We reconstructed a time-calibrated molecular phylogeny for *Croton* that included the greatest possible number of species, based on availability of sequence data. Sequences from GenBank were evaluated, and molecular markers were chosen for inclusion in the phylogenetic analyses if they were sampled for over 30 % of the available species. The result was a molecular data matrix that included 315 terminals and four markers. *Croton* was represented by 310 accessions, spanning all the major lineages, and outgroups – *Acidocroton*, *Astraea* and *Brasiliocroton* – were selected based on the most recent published phylogeny of *Croton* (van Ee *et al.*, 2011). The nuclear ribosomal internal transcribed spacer (ITS), the plastid *trnL-F* (*trnL* exon, intron and 3<sup>rd</sup> intergenic spacer), exon 9 of the coding low-copy nuclear gene *EMB2765* (*EMBRYO DEFECTIVE* 2765, At2g38770) and the mitochondrial protein-coding *rps3* gene were chosen, providing evidence from all three genomes. The concatenated dataset was first analysed using Bayesian phylogenetic reconstruction in MrBayes v3.2.1 (Ronquist *et al.*, 2012). To incorporate topological and branch length uncertainty in the comparative phylogenetic analyses, we also produced ultrametric trees from the Bayesian algorithms implemented in BEAST 1.8.0 (Drummond *et al.*, 2012) based on the combined DNA sequence data. [See [Supplementary Data](#) for detailed phylogenetic methods ([Methods S1](#)) and a list of species with GenBank accession numbers of the sequences used in this study ([Data S1](#)).]

### *Croton wood anatomy and character coding*

Microscopic wood anatomical data were scored for 289 xylarium specimens (most collected in the field for this study) from 171 species representing seven genera from tribe Crotonaeae. Specimens were prepared for microscopic observation and measured following standard microtechnique protocols (as in Berry and Wiedenhoef, 2004). We used the International Association of Wood Anatomists (IAWA) list of microscopic features for hardwood identification (IAWA Committee, 1989) as a starting point for character definitions, but adjustments were made according to wood anatomical variation specific to the tribe Crotonaeae. Originally, 53 discrete (binary or multistate) characters were scored, and all multistate characters were reduced to binary equivalents. A wood anatomical description

of *Croton* was generated based on 159 species and 272 specimens sampled from the genus. Simplified habit (shrub/tree) and habitat (mesic/xeric) character states were scored using information from herbarium specimen labels, literature (e.g. floras, taxonomic treatments) and our own field observations. This approach, although coarse, reduces uncertainty associated with accession and collection information for some xylarium specimens and provides field-based observations of plant habit and habitat. The binary classification of habit in *Croton* is typically reliable at the species level, and is inherently more amenable to a binary classification than is habitat, and thus we favour habit over habitat when the two factors are related to wood anatomical evolution. A list of all examined specimens and coded data for all 53 characters is found in [Wiedenhoeft \(2008\)](#).

The intersection between the sets of species included in the phylogenetic analysis and those available for anatomical characterization resulted in 102 taxa. Within this sampling, 17 wood anatomical characters are invariable and were removed. We treated the remaining 36 wood anatomical characters as independent and modelled each as a discrete character with binary states. Character definitions and scoring are explained in the Appendix.

#### *Inferring phylogenetic signal in wood anatomical characters*

All phylogenetic trees from the posterior distribution of trees generated with BEAST were trimmed to the number of species with wood anatomical data using the `drop.tip` function in the R package ‘ape’ ([Paradis et al., 2004](#)), which preserves topology and branch lengths. For each character, including habit and habitat, we estimated the character transition rate and calculated the likelihood of different models for discrete character evolution. This was done using the `fitDiscrete` function in the R package ‘geiger’ ([Harmon et al., 2008](#)) and on the maximum clade credibility tree (MCCT) from BEAST (see [Supplementary Data Methods S2](#), for detailed methodology).

A central aspect of our approach is the statistical non-independence among species’ character states because of their phylogenetic relatedness ([Felsenstein, 1985](#); [Revell et al., 2008](#)). We used ‘phylogenetic signal’ as an indicator for statistical non-independence. If a character exhibits phylogenetic signal, we expect the phylogeny to be helpful in explaining the distribution of the character states among the taxa. We assessed each character’s distribution for phylogenetic signal on the MCCT, to distinguish them from random trait distributions, using Pagel’s  $\lambda$  and Fritz and Purvis’  $D$  indices ([Fritz and Purvis, 2010](#); [Methods S2](#)).

#### *Ancestral character state reconstruction*

We reconstructed the character evolution for all characters exhibiting phylogenetic signal. We inferred their ancestral character states using both maximum likelihood, through the `ace` function in the R package ‘ape’ ([Paradis et al., 2004](#)), and Bayesian inference, which incorporates phylogenetic uncertainty into the ancestral state reconstruction, using the program BayesTraits V2.0 ([Pagel et al., 2004](#); [Pagel and Meade, 2013](#)) ([Supplementary Data Methods S3](#) presents a detailed methodology).

#### *Correlated and contingent wood anatomical evolution with habit and habitat*

We tested for correlated character evolution to detect evidence for adaptive or functional relationships between habit, habitat and wood anatomical features. Pagel and Meade’s Bayesian approach for testing correlated evolution of pairs of binary traits on phylogenetic trees ([Pagel and Meade, 2006](#)) was implemented via the Discrete module in the program BayesTraits V2.0 ([Pagel et al., 2004](#); [Pagel and Meade, 2013](#)). This approach accounts for uncertainty in both the model of trait evolution and the phylogeny (see [Supplementary Data Methods S4](#) for detailed methodology). We specifically tested for correlated evolution between habit, habitat and the binary wood traits exhibiting phylogenetic signal. Having discerned which wood features evolved in a correlated fashion with habit or habitat, we estimated transition rates underlying the correlated evolution.

We tested for contingent evolution between habit or habitat and each of the wood features that showed evidence of correlated evolution. Contingency tests detect if, when a pair of binary traits evolve in correlated fashion, the acquisition of the second state in a character is more probable when the other character has already acquired its second state, and vice versa. Using standard Markov chain Monte Carlo (MCMC) methods for Bayesian analyses like those outlined above, the Discrete BayesTraits method allowed us to constrain two transition rate parameters as equal, where the rate of gain of a character is the same in both states (e.g. in shrub and tree habit), and compare the restricted model with the unconstrained dependent model. Positive evidence indicates that the character state of habit or habitat (i.e. shrub vs. tree or xeric vs. mesic) influences the evolution of the wood feature, or vice versa ([Pagel, 1994](#)) (see [Supplementary Data Methods S5](#) for detailed methodology).

## RESULTS

#### *Phylogeny and node-age estimation*

Summary statistics for the data matrices and information about analyses, such as selected partitions and models of sequence evolution, are provided in the [Supplementary Data Table S1](#). Initial Bayesian phylogenetic analysis of the concatenated dataset (obtained in MrBayes) produced a well-resolved and strongly supported phylogeny [majority-rule consensus tree (MRCT), [Supplementary Data Fig. S1](#)], with a similar topology to the Bayesian phylogram of [van Ee et al. \(2011\)](#). The BEAST chronogram (MCCT, [Fig. S2](#)) displays widespread agreement to the MRCT, in overall topology and support values for most major deep clades, with some topological incongruences among shallower nodes within the well-supported clades. The weak support of some of these nodes emphasizes the need for a Bayesian approach to trait evolution that accounts for phylogenetic uncertainty.

The backbone topology of major lineages within *Croton* has strong support values in both the MRCT and the MCCT, with Bayesian posterior probabilities (PPs) of 1 ([Fig. 1](#); [Supplementary Data Figs S1, S2](#)). The four subgenera of *Croton* are each monophyletic ([Fig. 1](#)), with *C.* subgen. *Quadrilobi*

sister to all other subgenera. In turn, *C. subgen. Adenophylli* is sister to a clade consisting of *C. subgen. Croton*, the clade containing all Old World *Croton*, and *C. subgen. Geiseleria*. Geographical provenance of the species used in this study is found in [Supplementary Data Table S2](#). Most sections of *Croton* are recovered as monophyletic groups with strong support (PP > 95 %), although 12 are represented by a single accession (data not labelled). The clade corresponding to *C. subgen. Adenophylli* contains two well-supported subclades that represent two of the largest sections of the genus, *C. sects. Cyclostigma* and *Adenophylli* (PP = 100 %).

The estimated date for the *Croton* stem is 57.55 Ma and for the *Croton* crown 42.05 Ma. The four *Croton* subgenera arose within the next 6.9 Ma, where the estimated divergence of the Old World clade from its New World sister clade is 35.25 Ma ([Fig. 1](#); [Fig. S2](#)). These results represent updated estimates of divergence times for subgen. *Quadrilobi*, the core *Croton* clade and the subgenera that make up this clade: *Adenophylli*, *Croton* and *Geiseleria*.

#### Croton wood anatomy

Most wood anatomical characters are homoplasious across *Croton*, which was not unexpected, as it is a large and morphologically diverse genus ([Wiedenhoeft et al., 2003](#); [Riina, 2006](#)). Despite the wide range of wood anatomy in *Croton*, we present a generic wood anatomical description in [Supplementary Data Note S1](#). A total of 34 qualitative wood anatomical characters exhibited variation in their character states ([Table 1](#)). A list of *Croton* species with their coded character states for all qualitative woody features, including habit and habitat, is given in [Supplementary Data Table S3](#).

#### Inferring phylogenetic signal in wood anatomical characters

A trimmed version of the maximum-clade credibility phylogeny ([Fig. 1](#)), along with the character state per species for each particular trait, was used for fitting and comparing models of trait evolution. An equal-rates model (ER), in which a single parameter governs all transitions, was found to better fit the character state distribution of 14 wood anatomical characters and habit. An all-rates-different model (ARD), in which each rate is a unique parameter, was found to better fit the remaining 20 wood characters and habitat ([Supplementary Data Table S4](#)).

Tests for phylogenetic signal using Pagel's  $\lambda$  revealed that 24 of the 34 anatomical characters, habit and habitat, exhibit phylogenetic non-independence ([Table S4](#)). The Fritz and Purvis'  $D$  measure indicated that the distribution of 14 characters, habit and habitat, shows a moderate to high level of phylogenetic signal ([Table S4](#);  $D < 0.5$ ). Simulation tests showed that the phylogenetic patterns of laticifers and habit have the highest probabilities of significant phylogenetic signal (probability of estimating  $D$  under Brownian evolution = 0.97 and 0.78, respectively), whereas axial parenchyma in bands and crystals in true procumbent ray cells differ significantly from both Brownian expectation and random structure. Ten characters that revealed phylogenetic signal with Pagel's  $\lambda$  did not exhibit a strong signal with the Fritz and Purvis'  $D$  measure (i.e.

axial parenchyma in bands, axial parenchyma strand length, true procumbent ray cells, crystals in chambered upright/square ray cells, crystals in upright ray cells, crystals in chambered upright ray cells, crystals in nominal procumbent ray cells, crystals in true procumbent ray cells, crystals in non-chambered axial parenchyma cells, and helical thickenings in the vessel elements).

#### Ancestral character state reconstruction

Given the distribution of character states across topologies from our phylogenetic inference, we were able to provide a partial hypothesis of the wood anatomy of early *Croton*. The habit, habitat and wood anatomy of the reconstructed ancestral *Croton* are depicted in [Fig. 2](#), and are based on the ancestral character state reconstructions for the 14 characters exhibiting phylogenetic signal ([Supplementary Data Fig. S3](#)), and the marginal (from ape) and proportional (from BayesTraits) likelihoods of character states at selected nodes ([Supplementary Data Table S5](#)).

The most recent common ancestor of modern *Croton* is reconstructed as a tree with absent or indistinct growth rings, confluent-like axial parenchyma, nominal procumbent ray cells and disjunctive ray parenchyma cell walls ([Fig. 2](#)). Habitat, as well as seven wood anatomical characters (i.e. vessels in radial arrangement, fibre wall thickness, axial parenchyma strand length, the presence of true procumbent ray cells, perforated ray cells, crystals in upright ray cells and crystals in nominal procumbent ray cells), exhibit equivocal reconstructions, which Bayes factor tests were not able to resolve ([Table S5](#)). The remaining wood characters (i.e. aliform paratracheal axial parenchyma, axial parenchyma bands, axial parenchyma in marginal bands, laticifers, crystals in chambered ray cells, crystals in true procumbent ray cells, crystals in axial parenchyma, crystals in idioblasts and helical thickenings) were reconstructed as absent for the root node of *Croton*.

Most character state reconstructions at the four deep nodes representing the subgeneric clades did not vary from the reconstruction at the root node of *Croton*, except for vessels in radial arrangements, fibre wall thickness and laticifers. Although vessels in radial arrangement showed an equivocal reconstruction for the root of *Croton*, they are reconstructed as present for the root node of *C. subgen. Quadrilobi*, and absent for the root node of *C. subgen. Adenophylli* and *Geiseleria*. Thin fibre walls appear to be the ancestral state for the root nodes of *C. subgen. Adenophylli* and *Geiseleria*, whereas thick fibre walls are reconstructed at the root nodes of *C. subgen. Croton*. Laticifers are reconstructed as present in the root node of *C. subgen. Adenophylli* (occurring only at the base of *C. sect. Cyclostigma*).

Most characters exhibited multiple gains and/or losses across *Croton*, and these appeared to occur within each of the subgenera, after their lineages diverged. A noticeable pattern is observed within *C. subgen. Adenophylli*: for some characters (seven wood anatomical characters, in addition to habit and habitat), contrasting states are reconstructed at the root nodes of the two clades that make up the subgenus. The root node of *C. sect. Cyclostigma* is reconstructed as being a mesic tree with thin fibre walls, confluent-like and aliform paratracheal axial

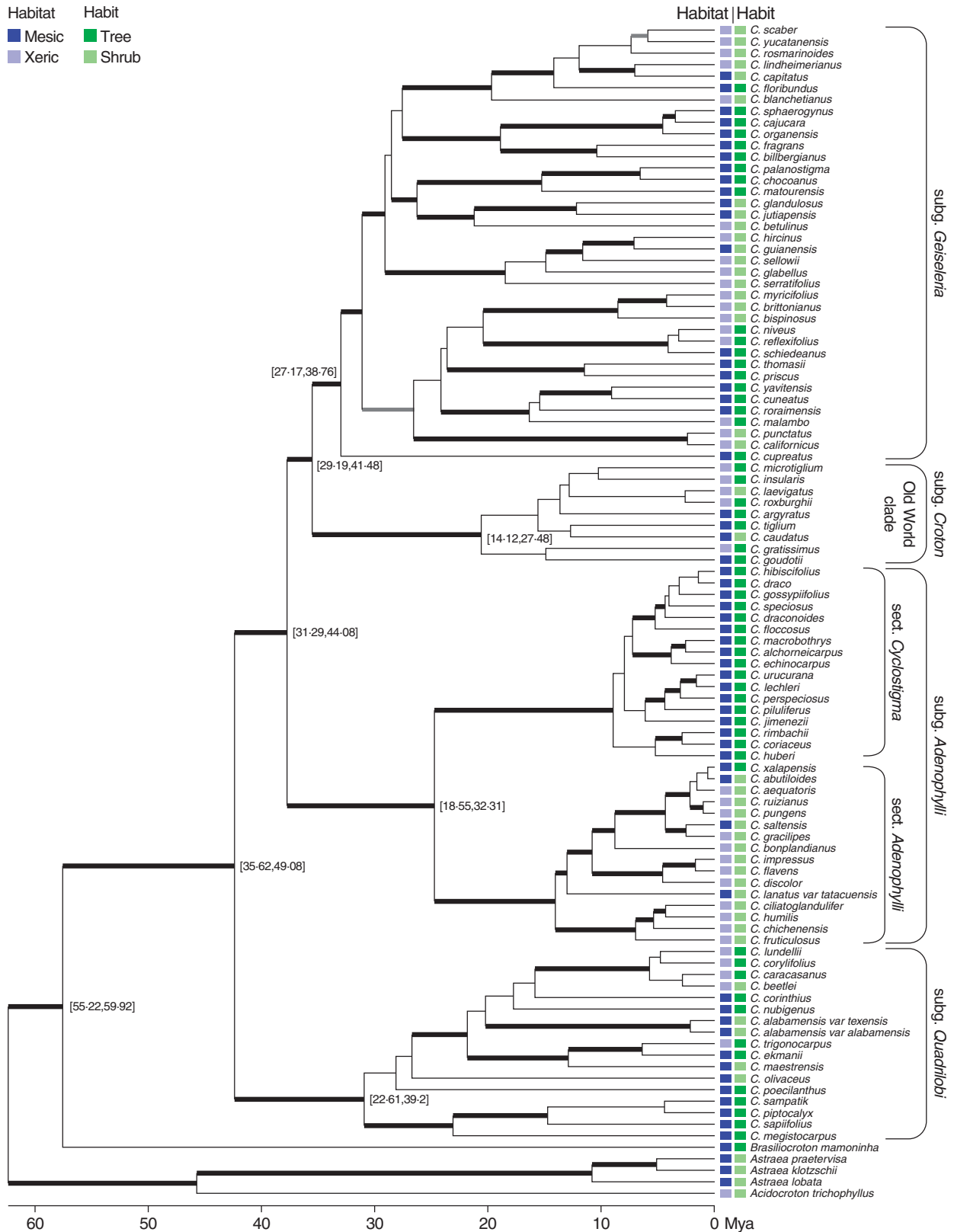


FIG. 1. Dated chronogram (the maximum clade credibility tree) inferred from the BEAST analyses of the 313-tip data set of *Croton* and outgroups. Only taxa for which there is wood anatomical data are included. Posterior probabilities  $\geq 0.9$  and  $< 0.95$  are indicated as thick grey branches, whereas probabilities  $\geq 0.95$  are indicated as thicker black branches. Numbers in brackets correspond to the 95 % highest posterior density (HPD) of the age of clades.

TABLE 1. List of the wood anatomical characters and character states that exhibited variation in their state within sampled species of *Croton*; habit and habitat included

	Trait	States
1	Habit	S=shrub, T=tree
2	Habitat	M=mesic, X=xeric
3	Growth rings	0=absent, 1=distinct
4	Wood ring porosity	0=diffuse, 1=ring-/semi-ring-porous
5	Vessels in radial arrangement	0=absent, 1=present
6	Vessels arranged in clusters	0=absent, 1=present
7	Vessels in radial groups of four or more	0=absent, 1=present
8	Vessel-ray pitting type	0=same in size and structure, 1=different in size and structure.
9	Fibre wall thickness	0=thin-walled, 1=thin- to thick-walled
10	Apotracheal axial parenchyma diffuse-in-aggregates	0=absent, 1=present
11	Paratracheal axial parenchyma vasicentric	0=absent, 1=present
12	Paratracheal axial parenchyma aliform	0=absent, 1=present
13	Confluent-like axial parenchyma	0=absent, 1=present
14	Axial parenchyma bands	0=absent, 1=present
15	Axial parenchyma in marginal bands	0=absent, 1=present
16	Axial parenchyma strand length	0=1–4 cells, 1=5 or more cells
17	Procumbent ray cells	0=absent, 1=present
18	Nominal procumbent ray cells	0=absent, 1=present
19	True procumbent ray cells	0=absent, 1=present
20	Perforated ray cells	0=absent, 1=present
21	Disjunctive ray parenchyma cell walls	0=absent, 1=present
22	Laticifers	0=absent, 1=present
23	Crystals in upright and/or square ray cells	0=absent, 1=present
24	Crystals in chambered upright/square ray cells	0=absent, 1=present
25	Crystals in upright ray cells	0=absent, 1=present
26	Crystals in chambered upright ray cells	0=absent, 1=present
27	Crystals in square ray cells	0=absent, 1=present
28	Crystals in chambered square ray cells	0=absent, 1=present
29	Crystals in procumbent ray cells	0=absent, 1=present
30	Crystals in nominal procumbent ray cells	0=absent, 1=present
31	Crystals in true procumbent ray cells	0=absent, 1=present
32	More than one crystal per ray cell	0=absent, 1=present
33	Crystals in axial parenchyma cells	0=absent, 1=present
34	Crystals in chambered axial parenchyma cells	0=absent, 1=present
35	Crystals in idioblasts	0=absent, 1=present
36	Helical thickenings in the vessel elements	0=absent, 1=present

parenchyma, true procumbent ray cells, laticifers and crystals in axial parenchyma cells, but without vessels in radial arrangement or perforated ray cells. The root node of *C. sect. Adenophylli* is reconstructed as being a xeric shrub, with thick fibre walls, without confluent-like or aliform paratracheal axial parenchyma, without true procumbent ray cells, laticifers or crystals in axial parenchyma, but with vessels arranged radially and perforated ray cells.

#### Correlated evolution

When considering phylogenetic relationships, the results of the BayesTraits analyses and Bayes factor tests identify a strong evolutionary correlation between habit and habitat (Bayes factor > 10), one of the strongest correlations between any two characters (Table 2), which is broadly in keeping with ecological factors influencing vegetation types and plant stature (O'Brien *et al.*, 2000; Reich *et al.*, 2003). For those wood anatomical characters correlated with both habit and habitat, given the correlation between those two factors, we assign the primary correlation to whichever correlation is more strongly supported, and presume that the more weakly supported character

is associated by virtue of the habit–habitat correlation. Interestingly, there are cases in which a wood anatomical character is correlated with either habit (wood ring porosity, aliform paratracheal axial parenchyma, confluent-like paratracheal axial parenchyma) or habitat (helical thickenings), but not with both (Table 2).

Six wood characters showed correlation with both habit and habitat with different strength of correlation, and only one, fibre wall thickness, showed equally strong correlation with both factors. Characters more strongly correlated with habit were axial parenchyma in bands, true procumbent ray cells, perforated ray cells and crystals in true procumbent ray cells. Characters more strongly correlated with habitat were vessel ray pitting type, axial parenchyma strand length and crystals in nominal procumbent ray cells (Table 2). [Results of Bayesian correlation tests between habit, habitat and wood anatomical characters are found in the Supplementary Data Table S6 (habit) and Table S7 (habitat).]

#### Tests of contingent evolution (wood characters appear in bold type for ease of reference)

Contingent patterns of evolution among habit, habitat and the wood anatomical characters are presented in flow-loop

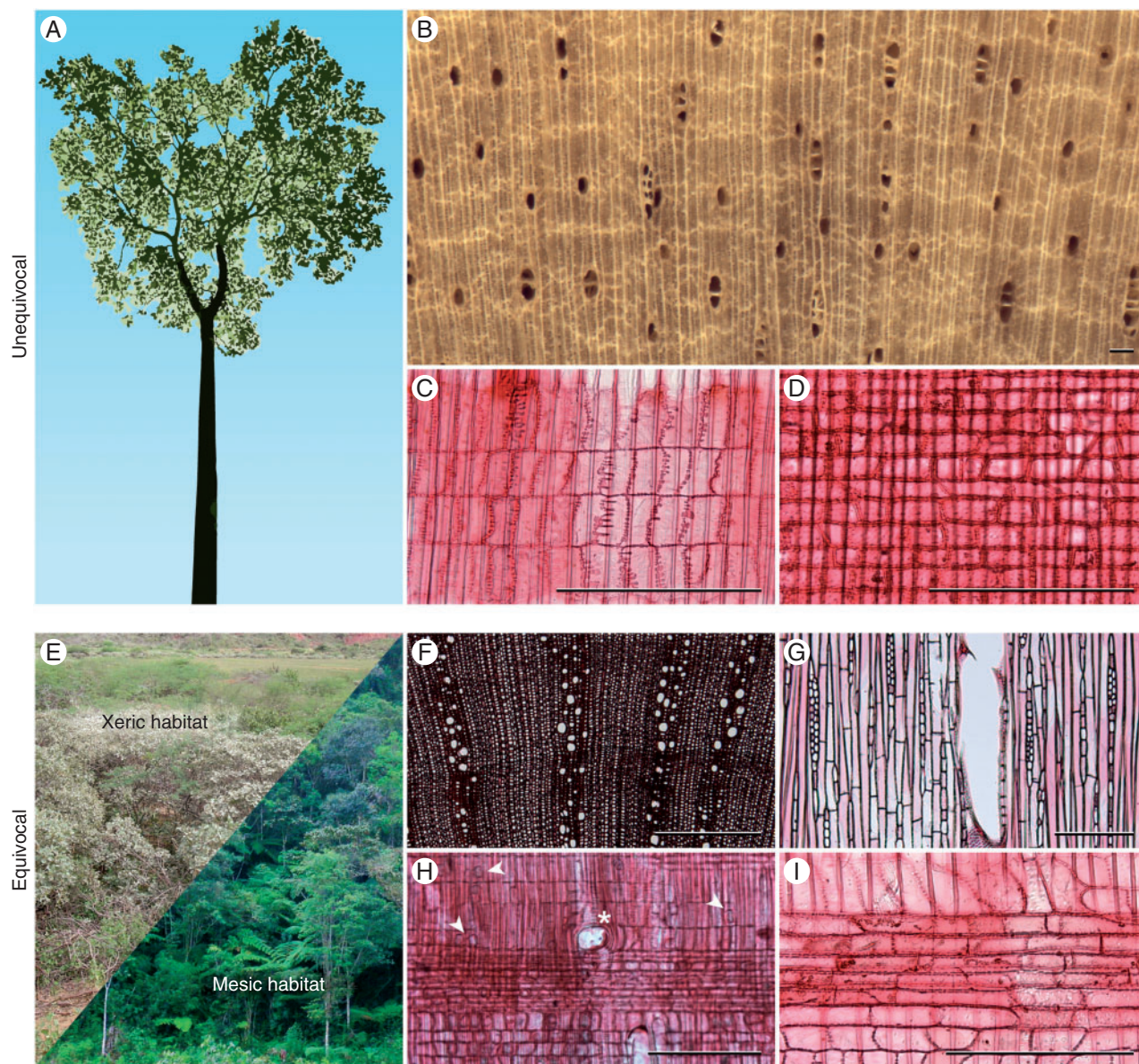


Fig. 2. Images (including light micrographs) of character states (unequivocal and equivocal) present in ancestral *Croton* inferred via ancestral state reconstruction. (A) *Croton macrobothrys* (Mata Atlantica, Bahia, Brazil) depicting the tree habit; (B) confluent-like axial parenchyma; (C) disjunctive ray parenchyma cell walls; (D) nominal procumbent ray cells; (E) mesic habitat depicted by *C. rimbachii* (Oxapampa Province, Pasco Region, Peru), and xeric habitat depicted by *C. thurifer* (Ucubamba Province, Amazonas Region, Peru); (F) vessels in radial arrangement; (G) > 4 cells per parenchyma strand; (H) perforated ray cells (asterisk) and crystals in upright ray cells (arrowheads); (I) true procumbent ray cells. Scale bars = 200  $\mu\text{m}$ .

diagrams in Figs 3–5. (Results of Bayesian contingency tests between habit, habitat and their correlated characters can be found in [Supplementary Data Tables S8 and S9](#).)

#### Habit and habitat

Figure 3 depicts the transitions between the different character-state-pairs (CSPs) of **habit** and **habitat**, under a model of dependent evolution. Transitions are shown as vectors with magnitude (rates) and direction. Each estimated transition rate is reported beside the arrow indicating the direction of

evolution between two CSPs and because transitions between two character states can be contingent on the state of the other character, each rate can be different.

Changes in habit are contingent on habitat, whereas transitions of habitat are not: shrubs are more likely to evolve (from trees) in a xeric habitat, and trees are significantly more likely to evolve from shrubs in a mesic environment. The CSP of mesic trees appears as a stable state, where inbound rates are of high magnitude and outbound rates are low, indicating rare transitions away from this CSP. Although habitat transitions were independent of habit, xeric trees appear as an

TABLE 2. Summary of results from Bayesian correlation tests between habit (and habitat) and wood anatomical characters using 1000 phylograms from the post burn-in period of the Bayesian phylogenetic analysis in BEAST

	Habit	Habitat
Growth rings	—	—
Wood ring porosity	**	—
Vessels in radial arrangement	—	—
Vessel ray pitting type	*	**
Fibre wall thickness	***	***
Paratracheal axial parenchyma aliform	*	—
Confluent-like axial parenchyma	**	—
Axial parenchyma in bands	**	*
Axial parenchyma in marginal bands	—	—
Axial parenchyma strand length	*	**
Procumbent ray cells	—	—
Nominal procumbent ray cells	—	—
True procumbent ray cells	***	*
Perforated ray cells	***	**
Disjunctive ray parenchyma cell walls	—	—
Laticifers	—	—
Crystals in chambered upright/square ray cells	—	—
Crystals in upright ray cells	—	—
Crystals in chambered upright ray cells	—	—
Crystals in chambered square ray cells	—	—
Crystals in nominal procumbent ray cells	*	**
Crystals in true procumbent ray cells	**	*
Crystals in axial parenchyma	—	—
Crystals in idioblasts	—	—
Helical thickenings	—	**
Habitat	***	—

\* = Bayes factor value > 2, taken as 'positive' evidence; \*\* = Bayes factor values > 5, taken as 'strong' evidence; \*\*\* = Bayes factor values > 10, taken as 'very strong' evidence.

unstable CSP; inbound rates are low whereas outbound rates are high.

#### Habit and wood anatomical characters (Fig. 4)

**Ring or semi-ring porosity** evolves in shrubs but does not influence shifts in habit, and is comparatively rare in *Croton*. **Vessel-ray pitting type** influences habit shifts, with same size intervessel and vessel-ray pits predicting the evolution from a tree to a shrub, and vessel-ray pits of different size than intervessel pits predicting the evolution from a shrub to a tree. **Axial parenchyma strand length** shows a similar pattern to vessel-ray pitting, where the evolution from shrub to tree was more likely to occur in the presence of strands longer than five cells, and the evolution from a tree to a shrub in the presence of strands with four or fewer cells. Within habits, gain or loss of the character is similarly likely. Most *Croton* have **thick-walled fibres**, which

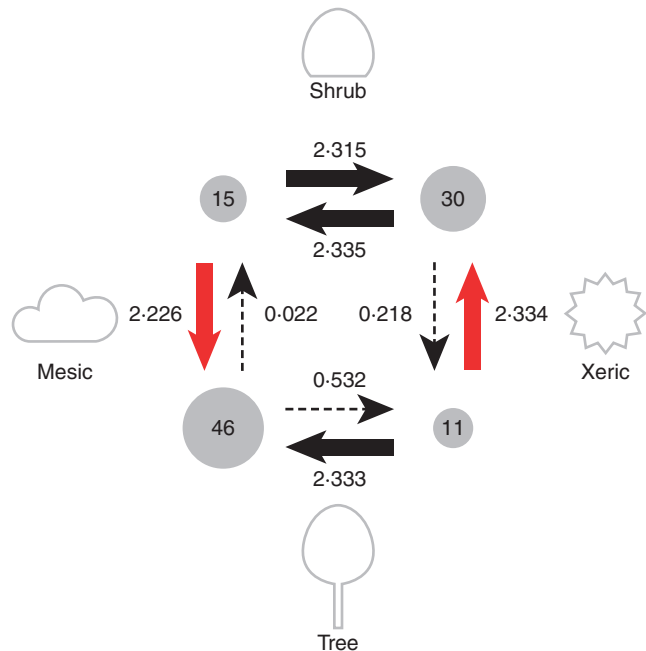


FIG. 3. Flow-loop diagram showing significant (solid arrows) and non-significant (dotted arrows) transitions in the correlated evolution between habit and habitat, as well as cases of contingent evolution (red arrows), where the state of one character has a significant influence on the evolution of the other. Thick black arrows indicate transition rates that have a low posterior probability of being zero (Z-score > 5%) and for which alternative model tests rejected a hypothesis that the given transition rate has a value of zero in favour of a non-zero value across all examined trees. Thin black arrows highlight transitions that have an intermediate posterior probability of being zero (Z-score 10–80%) and for which alternative model tests failed to reject a hypothesis that the given transition rate has a value of zero across all examined trees. Dashed arrows indicate transition rates that have a high posterior probability of being zero (Z-score < 95%) and for which alternative model tests failed to reject a hypothesis that a given transition rate has a value of zero across examined trees. Red arrows highlight results of contingency tests, where alternative model tests rejected the hypothesis that the two transition rates of one character are equal, or independent of the second character, in favour of different dependent rates across examined trees. The size of each circle is proportional to the percentage of sampled species that are found in each character state combination.

were most likely to evolve in trees, and with shrubs evolving from trees which possess thick-walled fibres. Shrubs and trees show no difference in gain or loss of **aliform parenchyma**, but for a shrub to evolve into a tree in *Croton*, the presence of aliform parenchyma seems to be necessary. For both **confluent-like axial parenchyma** and **axial parenchyma in bands**, the evolution of these characters occurs almost exclusively in trees, with little influence of the presence or absence of the character on habit shifts. A shrub is much more likely to evolve into a tree in the presence of **crystals in nominal procumbent ray cells**, both trees and shrubs are more likely to lose than to gain this character, and the absence of the character does not influence habit shifts. Despite the presence of **crystals in true procumbent ray cells** being rare in the *Croton* we sampled, we recovered evidence for contingent evolution between the character and habit. Since the direction and magnitude of the transitions associated with their contingent evolution are similar to, but



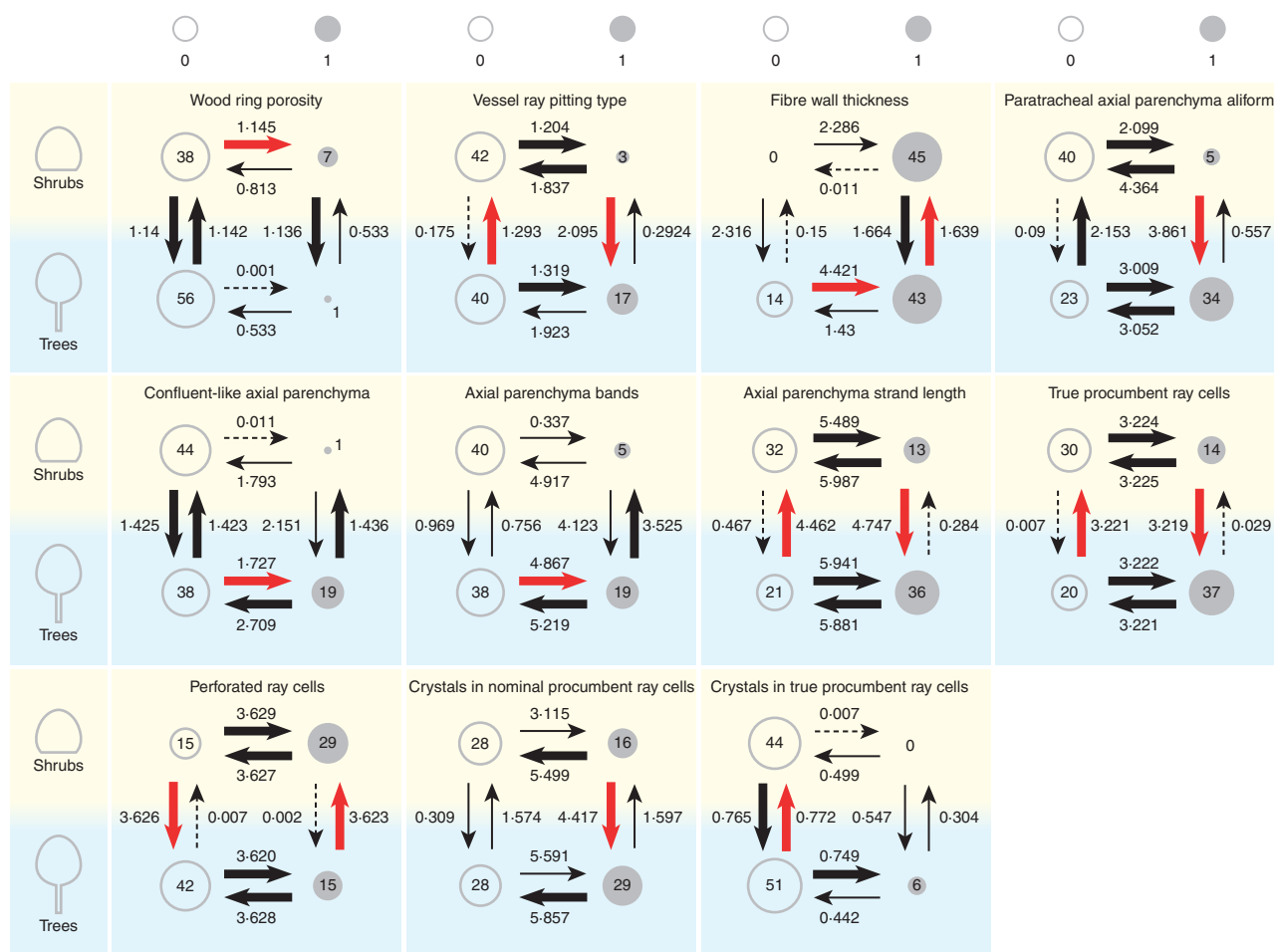


FIG. 4. Flow diagrams showing significant (solid arrows) and non-significant (dotted arrow) transitions in the correlated evolution between habit and 11 wood anatomical characters, as well as significant cases of contingent evolution (red arrows), where the state of one character has a significant influence on the evolution of the other. 0 and 1 on top correspond to character states that are absent or present, respectively, for all characters except wood ring porosity (diffuse or ring-/semi-ring-porous), vessel-ray pitting type (same in size and structure/different in size and structure), fibre wall thickness (thin-walled/thin- to thick-walled) and axial parenchyma strand length (1–4 cells/5– more cells).

weaker than, that of the true procumbent ray cell character and habit, it is most likely an analogue to part–whole correlation. Thus, we suspect that this relation may be artefactual and is only reflecting the underlying contingent relationship. For **true procumbent ray cells** and **perforated ray cells**, the gain or loss of the character is not influenced by plant habit, but transitions between habits are contingent on the character states. In the case of true procumbent ray cells, trees are more likely to transition to a shrub in their absence, whereas shrubs are more likely to transition to a tree once true procumbent ray cells have been gained. Perforated ray cells show a converse relationship, with shrubs being more likely to transition to a tree in their absence, and trees becoming shrubs when perforated ray cells are present.

#### Habitat and wood anatomical characters (Fig. 5)

Not only are there fewer wood anatomical characters correlated with habitat, but in general, the number of contingent evolutionary relationships was fewer.

For **vessel-ray pitting**, **axial parenchyma in bands** and **crystals in true procumbent ray cells**, character state gains are significantly more likely to evolve in mesic than in xeric habitats. In both mesic and xeric habitats, **fibres** are more likely to evolve to the thick-walled condition, and transitions between habitats only take place once fibres are thick-walled. With **axial parenchyma strand length** and **true procumbent ray cells**, the absent character state is necessary for mesic to xeric habitat transitions, and no other transitions show contingent evolution. In the presence of **perforated ray cells**, transitions from mesic to xeric habitat are significantly more likely to occur, and no other transitions show contingent evolution. **Crystals in nominal procumbent ray cells** show correlated but not contingent evolution and so nothing of consequence can be inferred from these data. **Helical thickenings** in vessel elements evolve in mesic habitats significantly more frequently than in xeric habitats, and the transition rates for this character pair are the highest of any studied here. Also, within the mesic habitat, the rate for loss of the character is almost equal to rate of gain, whereas

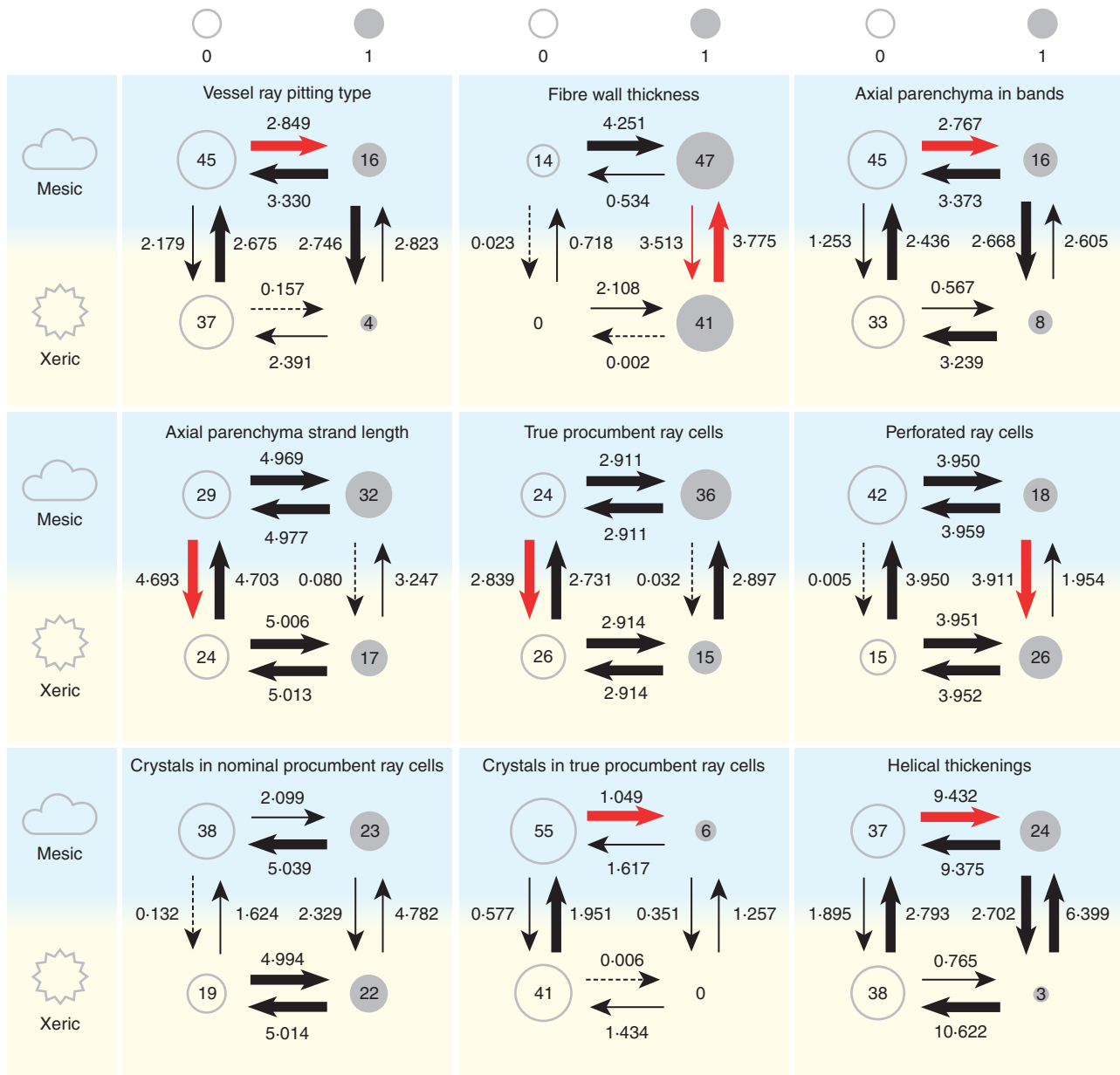


FIG. 5. Flow diagrams showing significant (solid arrows) and non-significant (dotted arrows) transitions in the correlated evolution between habitat and nine wood anatomical characters, as well as significant cases of contingent evolution (red arrows), where the state of one character has a significant influence on the evolution of the other. 0 and 1 on top correspond to character states that are absent or present, respectively, for all characters except vessel-ray pitting type (same in size and structure/different in size and structure), fibre wall thickness (thin-walled/thin- to thick-walled) and axial parenchyma strand length (1–4 cells/5– more cells).

within the xeric habitat the rate of character loss is considerably higher than the rate of gain.

## DISCUSSION

### Phylogeny and node age estimation

Our results are fundamentally congruent with previous studies examining the relationships within *Croton* (Berry et al.,

2005b), especially with the topology of the most recent phylogeny (van Ee et al., 2011) and the node ages reported by van Ee et al. (2008). In this study, we present the largest phylogenetic analysis of *Croton* to date and estimate node ages to establish a distribution of ultrametric trees from which to reconstruct ancestral character states and use this as a tool to explore wood anatomical evolution. These trees support van Ee et al.'s (2008) reported node ages for subgenus *Quadrilobi* and the core *Croton* clade, and provide a broader taxon sampling to confirm

clade ages for subgenera *Adenophylli*, *Croton* and *Geiseleria*, and further show these clades diverging in a comparatively narrow time-frame of 6–7 Myr.

#### Croton wood anatomy

The wood anatomical diversity in *Croton* is high, with a wide range in character states across a large number of characters, which is one reason why the genus is so well suited for this type of exploration. In addition to demonstrating high wood anatomical diversity, our results confirm an earlier report of extensive wood anatomical homoplasy across the genus (Wiedenhoef, 2008), as well as homoplasy in both habit and habitat. From a purely systematic context, homoplasy in *Croton* wood anatomy would be problematic, but when considering implicitly functional questions about the evolution of wood anatomical diversity related to habit and habitat, such homoplasy is in fact necessary for robust and non-spurious inference of associations (replicated co-distribution or replicated bursts, *sensu* Maddison and FitzJohn, 2015).

#### Inferring phylogenetic signal in wood anatomical characters

Because habit and habitat showed strong phylogenetic signal, we restricted the set of wood anatomical characters to those that also show phylogenetic signal and where selection and trait conservatism could have had a role in shaping their character state distribution. Thus, the means by which phylogenetic signal was inferred is central to our analysis. Using two approaches allowed us to detect characters whose distribution, although deviating from both random pattern and strict Brownian motion (Fritz and Purvis'  $D$ ), still revealed some phylogenetic non-independence (Pagel's  $\lambda$ ). Instances of characters exhibiting phylogenetic clustering also occurred, particularly within *C.* subgen. *Adenophylli*. Still, not all characters with phylogenetic signal showed correlated evolution with habit or habitat. In the case of the distribution of laticifers across *Croton*, despite having a high degree of phylogenetic signal (Table S4), the character was gained only once in the phylogeny, reconstructed as an underlying synapomorphy for *C.* sect. *Cyclostigma*, and so does not show a meaningful functional correlation with either habit or habitat.

#### Ancestral character state reconstruction

The ancestor of *Croton* is unequivocally reconstructed as being a tree, with the shrub habit evolving multiple times and only two reversions to trees (i.e. *C. floribundus* and *C. xalapensis*). Petit and Hampe (2006) showed that trees tend to have higher levels of genetic diversity compared to shrubs, and appear to be more capable of rapid adaptation to new conditions, which is consistent with the evolution of habit in *Croton* as inferred here. Despite the strong correlation between habit and habitat, reconstructions of habitat at the root node of *Croton* and for the four subgenera are equivocal. Based on the distribution of xeric and mesic taxa across the phylogenies, at least one habitat reversion is present in each of the subgeneric clades.

#### Correlated and contingent wood anatomical evolution with habit and habitat (wood characters appear in bold type for ease of reference)

##### Habit and habitat

With the common ancestor of all the subgenera of *Croton* reconstructed as a tree, the flow-loop diagram shown in Fig. 3 provides insight into the relationships between **habit** and **habitat** in the genus. Given that extant *Croton* are not solely mesic trees, and indeed that there are several clades within the phylogeny that are almost exclusively xeric shrubs, the flow-loop diagram shows that transitions to the shrub habit would almost exclusively have come through the unstable CSP of xeric trees. This is particularly interesting because the ancestral character state reconstruction for habitat was equivocal, but if extant *Croton* have evolved according to the flow-loop diagram, the only path that can result in the relatively large numbers of xeric shrub species (i.e. 30) is through xeric trees, which suggests a required ancestral character state of xeric conditions for habitat, even though our reconstruction of ancestral habitat is equivocal. Figure 3 also shows the most probable evolutionary route from xeric shrubs to mesic trees as going through mesic shrubs first. There are two taxa in the phylogeny (Fig. 1) that show this transition, *Croton floribundus* and *C. xalapensis*. *Croton floribundus* is found on a moderately long branch in *C.* subgen. *Geiseleria*. Maximum-likelihood reconstructions for habit and habitat (Fig. S3) show that the common ancestor of *C. floribundus* and its sister clade was probably a xeric shrub, suggesting that along the branch leading to *C. floribundus* both habitat and habit shifted, but with no way to infer the order of transitions, other than by assuming the transitions shown in Fig. 3. *Croton xalapensis* is a mesic tree found in a lineage of xeric shrubs, *C.* sect. *Adenophylli*, but in this case the phylogeny and character state transitions (Fig. 1) closely parallel the pathway in Fig. 3. *Croton xalapensis* is sister to *C. abutiloides* and maximum likelihood (Fig. S3) reconstructs their common ancestor as a mesic shrub. *Croton abutiloides* remained a shrub, but tree habit evolved along the branch to *C. xalapensis*, demonstrating the order of evolutionary transition shown in the flow-loop of Fig. 3; first a transition from xeric shrub to mesic shrub, and then to mesic tree.

##### Habit and wood anatomical characters

*Croton* is a mostly tropical and subtropical genus, and **ring or semi-ring porosity** is predominantly a cool temperate phenomenon in woods worldwide. The small proportion of ring-porous taxa found in *Croton* is of interest, given that this trait appears to have evolved multiple times across the phylogeny, and the temperate-most taxa are not the only species in which this evolved.

We know of no prior literature identifying any functional relationship between **vessel-ray pitting type** and habit or habitat, but hypotheses about transfer-cell/contact-cell functions in rays (Murakami et al., 1999) may be relevant analogues possibly implicating nutrient transfer between rays and vessels, and/or relating to potential roles for parenchyma cells (see also aliform and confluent-like axial parenchyma, below) in contact with

vessels altering the osmotica of the transpiration stream (Nardini *et al.*, 2012) which could be advantageous in plants with long hydraulic path lengths (in this case, trees). Our character state definition (see Appendix) includes a range of vessel-ray pit types that may be worth exploring separately to determine if one or more sub-types are the primary drivers of the pattern uncovered here.

Evidence for a possibly functional relationship between **axial parenchyma strand length** and habit is based on the fact that when a given length of axial parenchyma is divided into  $n$  cells, there are  $n$  possible separate physiological units (cells) available for specialization of function. If axial parenchyma, especially paratracheal parenchyma, plays an active role in regulating flow in the vessels, enhanced ability for functional specialization might be beneficial to the plant. The flow-loop diagram (Fig. 4) demonstrates that variations in axial parenchyma strand length drive changes in habit, and not vice versa.

The ancestral character state reconstruction was equivocal for **fibre wall thickness**, which could be related to the high magnitude of the transition rate between trees with thin-walled fibres to trees with thick-walled fibres, as shown in Fig. 4. The flow-loop diagram shows that thick-walled fibres evolve first in trees, and then trees with thick-walled fibres evolve into shrubs, and all extant shrubby *Croton* studied have thick-walled fibres. We know that the ancestral *Croton* was a tree, which implies a certain minimum precipitation from a vegetation standpoint, but it does not do anything to help infer fibre wall thickness, as the woods with some of the thinnest-walled (e.g. some *Alstonia*) and thickest-walled (e.g. *Krugiodendron*) fibres in the world are found in tree species.

The fact that the presence of **aliform parenchyma** seems to be necessary for a shrub to evolve into a tree is particularly striking in the context of the hypothetical role of parenchyma in regulating the osmotica of the transpiration stream (Nardini *et al.*, 2012). On average, trees have longer hydraulic path lengths than shrubs, and enhanced ability to respond to hydraulic dynamics could present a functional advantage for the evolution of tree habit, as noted for axial parenchyma strand length.

**Confluent-like axial parenchyma and axial parenchyma in bands** are similar in that they form comparatively long, tangentially orientated networks of parenchyma cells not directly associated with the vessels. In larger diameter taxa (e.g. trees) both radial ('interannual') and tangential ('intraannual') communication and physiological integration are presumably required. The radial system provisionally provides this function for radial transport (at least up to the sapwood–heartwood boundary, when present) but communication within a growth increment (whether distinguishable or not) would have to be by tangentially orientated connections, which could be provided by these tangentially orientated parenchyma patterns. Growth increments in trees have greater circumference than in shrubs, so trees might be expected to have greater need of a devoted tangential system of parenchyma. The validity of this conjecture should be tested in other taxa.

The presence of **crystals** in woods from xeric habitats is widely known, and crystals are presumed to deter herbivory in other parts of the plant body, although it is not clear what wood herbivores restricted to xeric habitats might drive the evolution of crystals in wood. Our data are the first we know of to show a relationship between the evolution of habit and crystals in

wood ray cells, and in the absence of a clear hypothesis for anti-herbivory, other potential functional roles or causes should be considered (e.g. controlling cellular osmotica in water-limited conditions).

**True procumbent ray cells and perforated ray cells** are the two most interesting wood anatomical characters that show contingent evolution with habit. Based on their aspect ratio and lack of pitting to vessels, true procumbent ray cells are implicated as 'isolation' cells (*sensu* Braun, 1967; Murakami *et al.*, 1999, Morris and Jansen, 2016). Their prominent radial elongation is consistent with their gain being a prerequisite for remaining a tree (Fig. 4). Because trees are larger diameter than shrubs and true procumbent cells are several times radially more elongated than nominal procumbent ray cells, the scaling of effective radial stem diameter (e.g. sapwood width) and ray cell length may be maintained by evolving these cells. Figure 4 shows that a tree without them either will gain them, or is likely to become a shrub. Similarly, a shrub with true procumbent ray cells is likely either to lose them or to evolve into a tree, but a shrub without true procumbent ray cells virtually never evolves into a tree. These patterns would have been impossible to discover without breaking from traditional character definitions and adapting the character coding for the range of variability observed in *Croton* wood anatomy, because **procumbent ray cells** (the traditional definition) and **nominal procumbent ray cells** did not show contingent evolution with habit. This issue of character definitions, whether based on one of the IAWA lists (IAWA Committee, 1989; Richter *et al.*, 2004) or other attempts to standardize nomenclature (Ruffinatto *et al.*, 2015), is a key component of a study like this. It has been eloquently argued that such lists are not the place to look for functional traits (Olson, 2005), but they are clear starting points (Baas *et al.*, 2016; Beeckman, 2016), especially if researchers can properly scale their character definitions to the organisms and questions at hand. The vagaries of character definition are much less problematic when using phylogenetic comparative methods to analyse continuous quantitative data.

**Perforated ray cells** are individual ray cells that, instead of only showing pitting to adjacent parenchyma cells, vessel elements or fibres, bear perforation plates to adjacent vessels. Typically these perforations are in the radial faces of the cells, giving rise to a putatively open tangential pathway between vessels through a ray. Based on the transitions seen in Fig. 4, in *Croton* the only route to the shrub habit is by a tree acquiring perforated ray cells, and then the only path from a shrub to a tree is in their absence. Ancestral *Croton* was a tree, and the shrub habit arose multiple times, but the ancestral character state reconstruction for **perforated ray cells** was equivocal. The gain of perforated ray cells in trees to then evolve into the shrub habit is a novel finding, and by its very nature negates the potential argument that vessels in shrubs are smaller and more abundant and thus make more likely the presence of perforated ray cells, because perforated ray cells must first be gained in trees. This strongly suggests a functionally advantageous role for these cells in shrubs, and further suggests a functional cost for maintaining them in trees. If this relationship is borne out in other taxa, it has the potential to encourage altogether new thinking about 'anomalously' perforated elements in wood, as they may be indicators not of structural or

developmental anomaly, but rather an ongoing evolutionary transition ‘caught in the act’.

#### *Habitat and wood anatomical characters*

Given that habit and habitat are strongly correlated, we consider it plausible that some of the relationships we found between habitat and wood anatomical characters may be a result of this strong habit–habitat correlation. The only wood anatomical character correlated with habitat but not with habit is **helical thickenings**, which are more likely to evolve in a mesic habitat, as compared to a xeric one, but which can also be lost at high rates in both types of habitat. These results seem to contradict earlier findings, where helical thickenings tend to be more abundant in areas that are drier (Webber, 1936; Carlquist, 1966). However, much like the discussion of ring and semi-ring porosity associated with habit, helical thickenings in vessel elements are more typical of higher-latitude taxa and are comparatively infrequent in the tropics (Nair, 1987; Wheeler *et al.*, 2007). Explicit exploration of the presence and role of helical thickenings in other taxa should be conducted with phylogenetic comparative methods to test whether such contingent evolution found in *Croton* is common or rare in wood at large. This would contribute meaningful comparative data to the rich and growing literature on hydraulic architecture of woody plants, but does not speak directly to the function of this character in wood.

The character definition used here included helical thickenings present throughout the body of the vessel element as well as helical thickenings restricted to the vessel element tails. We consider it highly likely that the hydraulic and functional roles of these two types of helical thickening also differ based on the disparity in distribution within the cell. Helical thickenings in the body of the vessel element are in the primary long-distance flow-path in the transpiration stream of hardwoods. Jeje and Zimmerman (1979) showed increased flow rate in vessels with wall sculpturing, and Wang (2006) solves the problem of helical flow in conditions with low Reynolds numbers (viscous fluids or tubes of small diameter). Testing the spacing and pitch of helical thickenings relative to conduit diameter and the predictions of helical flow (Wang, 2006) should prove a straightforward way to test this hypothesis.

It is difficult to implicate helical thickenings in the tails of vessel elements with a compelling role in the safety or efficiency of long-distance bulk flow because the tails are not located in the flow-path. One view of helical thickenings in the tails of vessel elements could be as a relict, devoid of functional relevance, but this would require the loss of helical thickenings from the body of the element. If there are cases of gain of helical thickenings in the tails of vessels elements without their presence throughout the cell, a functional value could be implicated. Based on contact angle measurements of water menisci in pit chambers (Zwieniecki and Holbrook, 2000) a role in vessel refilling following embolism could be implicated, because it is unlikely that embolism could cause vessel element tails to empty of water. Changing the osmotica of the comparatively tiny volumes of vessel element tails would require much less solute than the entire vessel lumen, and the wettability of the cell wall could facilitate flow into the lumen at large. This is

speculative, but would reconcile the disparate distributions of this trait in vessels with known physical phenomena.

## GENERAL CONCLUSIONS

We identified a number of relationships among habit, habitat and wood anatomical characters in *Croton*, suggestive of new functional relationships. By using reconstructed ancestral character states, especially the strong support for ancestral *Croton* being a tree, and quantitative estimates of correlated and contingent evolution, we were able to show that specific pathways were required in the evolution of shrubby clades. Specifically, the ancestral *Croton* tree had to first evolve same-size vessel-ray pitting, thick-walled fibres, fewer than five cells per parenchyma strand and perforated ray cells before evolving the shrub habit. Resolving these evolutionary pathways would not be possible using these methods in isolation. Future work in other taxa ideally would mine all available characters, but should include explicit tests of correlated and contingent evolution of habit perforated ray cells, habit true procumbent ray cells, and habitat helical thickenings in vessel elements. Taxa selected for additional study should be species-rich woody or mostly woody clades with a diversity of habits, such as *Eugenia* (Myrtaceae), *Psychotria* (Rubiaceae), *Schefflera* (Araliaceae) or *Ardisia* (Primulaceae), or revisiting *Ilex* (Aquifoliaceae), or any of a number of other genera or families. As we did here, character definitions for wood anatomical features should be calibrated based on the range of variability within the taxa in question, and in the context of the hypotheses to be tested. Operating on large wood anatomical data sets with statistical and phylogenetic rigor is a step toward a Big Data-style approach to evolutionary wood anatomical inference that capitalizes on the strengths of hypothesis-driven approaches, but also allows testing for as-yet-novel relationships that, while beyond the scope of current functional understanding, may suggest avenues for future reasoning and experimentation.

## SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Data S1: list of taxa included in this study along with GenBank accession numbers. Methods S1: phylogenetic inference. Methods S2: examining wood anatomy evolution. Methods S3: character state reconstruction. Methods S4: testing for correlated evolution. Methods S5: tests of contingent evolution. Table S1: overall molecular variation for each locus/data set. Table S2: list of *Croton* species included in the analyses along with their geographical provenance. Table S3: list of *Croton* species along with coded character states for all woody features, including habit and habitat. Table S4: evolutionary (Markov) models that are most likely to have generated the current distribution of wood anatomical character states across the *Croton* phylogeny, along with results from Pagel’s  $\lambda$  and Fritz & Purvis’  $D$  tests for phylogenetic signal of each of the characters. Table S5: marginal (from ape) and proportional (from BayesTraits) likelihoods of character states at selected nodes for each of the 26 characters that revealed phylogenetic signal across the *Croton* phylogeny. Table S6: results of Bayesian correlation tests

between habit and wood anatomical characters, and habit and habitat. Table S7: results of Bayesian correlation tests between habitat and wood anatomical characters. Table S8: results of Bayesian contingency tests between habit and habitat, and habit and wood anatomical characters. Table S9: results of Bayesian contingency tests between habitat and correlated wood anatomical characters. Figure S1: majority-rule consensus tree (MRCT) summarizing Bayesian analysis of the concatenated DNA dataset (obtained through MrBayes). Figure S2: maximum clade credibility tree (MCCT) summarizing Bayesian analysis of the concatenated DNA dataset (obtained through BEAST). Figure S3: ancestral character state reconstructions of habit, habitat and wood anatomical characters across the MCCT phylogeny of *Croton*. Note S1: wood anatomical description of *Croton*.

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## APPENDIX

### CHARACTER DEFINITIONS

- Habit 0 = subshrub to shrub, 1 = tree  
Habit was coded based on the general appearance of the plant when collected and confirmed using information from herbarium labels, floras and other taxonomic literature. Habit is typically a stable and intrinsic characteristic in most *Croton* (van Ee et al., 2011).
- Habitat was coded in two states 0 = mesic, 1 = xeric  
Coding habitat as a binary character necessarily involves simplifying a complex continuum of environmental conditions. To best reflect subtleties in site characteristics, determinations for this feature were made mostly by first hand knowledge of R. Riina, P. Berry and B. van Ee, with additional information taken from herbarium labels, flora and other references.
- Growth rings boundaries 0 = indistinct or absent, 1 = distinct  
Distinct growth rings exhibit a structural change at the boundaries between them, usually including a change in fibre wall thickness, vessel diameter and marginal parenchyma.

- Wood ring porosity 0 = diffuse, 1 = semi-ring- to ring-porous

Diffuse-porous wood has vessels of more or less the same diameter throughout the growth ring. In semi-ring- and ring-porous woods, the vessels in the earlywood are larger than those in the latewood of the previous growth ring. In semi-ring-porous wood they gradually change to narrower vessels in the latewood of the same growth ring or, the wood exhibits a distinct ring of closely spaced earlywood vessels that are not noticeably larger than the latewood vessels. In ring-porous wood, distinctly larger earlywood vessels form a well-defined zone or ring with an abrupt transition to the latewood of the same growth ring.

- Vessels in radial arrangement 0 = absent, 1 = present  
Vessels arranged radially or oblique.
- Vessels arranged in clusters 0 = absent, 1 = present  
Groups of three or more vessels having both radial and tangential contacts, and of common occurrence.
- Vessels in radial groups of four or more 0 = absent, 1 = 4 or more common  
Radial clusters of four or more adjacent vessels are of common occurrence.
- Vessel-ray pitting type 0 = same in size and structure, 1 = different in size and structure

These character states are different from those in the IAWA list (1989), but reflect a more targeted set of definitions specific to this work. Hence, pits between a ray cell and a vessel element can be: similar to intervessel pits in size and shape throughout the ray cell; or smaller than intervessel pits, larger than intervessel pits, unilaterally compound (where pits abut two or more smaller pits in the adjacent cell), or simple to linear. In the absence of *a priori* functional hypotheses for these vessel-ray pitting types, we have grouped them in a single character state.

- Fibre wall thickness 0 = thin-walled, 1 = thin- to thick-walled  
The classes of fibre wall thickness are based on the ratio of lumen to wall thickness: for thin-walled fibres the lumen is 3 or more times wider than the double wall thickness; in thin- to thick-walled fibres the lumen is less than 3 times the double wall thickness, and distinctly open; and for thick-walled fibres the lumen is almost completely closed.

- Apotracheal axial parenchyma diffuse-in-aggregates 0 = absent, 1 = present  
Axial parenchyma diffuse-in-aggregates refers to parenchyma strands grouped into short discontinuous tangential or oblique lines.

- Paratracheal axial parenchyma vasicentric 0 = absent, 1 = present  
Paratracheal parenchyma is axial parenchyma associated with the vessels elements. Vasicentric parenchyma refers to parenchyma cells that form a complete circular to oval sheath around a solitary vessel or vessel cluster.

- Paratracheal axial parenchyma aliform 0 = absent, 1 = present  
Aliform parenchyma is found surrounding, or to one side of, the vessel and with lateral extensions.

- Confluent-like axial parenchyma 0 = absent, 1 = present



Confluent-like axial parenchyma refers to coalescing irregular bands of mostly apotracheal parenchyma, the pattern of which, macroscopically, is confluent-like in its arrangement.

**14.** Axial parenchyma bands 0 = absent, 1 = present

Axial parenchyma in bands of up to three cells wide, that may be in contact but not associated with vessels (apotracheal). Bands may be wavy, diagonal, straight, continuous or discontinuous.

**15.** Axial parenchyma in marginal bands 0 = absent, 1 = present

Parenchyma bands that form a more or less continuous layer of variable width at the margins of a growth ring or are irregularly zonate.

**16.** Axial parenchyma strand length 0 = 2 to 4 cells per parenchyma strand, 1 = 5 or more cells per parenchyma strand

Parenchyma strands are a series of axial parenchyma cells formed through transverse division(s) of a single fusiform cambial initial.

Ray composition was coded somewhat differently from the IAWA (1989) list definitions, due to the subtleties of *Croton* ray structure.

**17.** Procumbent ray cells 0 = absent, 1 = present

Procumbent ray cells are defined as a ray parenchyma cell with its radial dimension longer than its axial dimension. In this study, procumbent cells were broken into two categories: nominal procumbent cells and true procumbent cells.

**18.** Nominal procumbent ray cells 0 = absent, 1 = present

Nominal procumbent cells are defined as those cells with its radial dimension between 150 and 250 % of the axial dimension; roughly brick-shaped in their aspect ratio.

**19.** True procumbent ray cells 0 = absent, 1 = present

True procumbent cells are defined as those cells in which the radial extent is 300 % or more of the axial extent of the cell. True procumbent cells are often lower than nominal procumbent cells as well, and often have distinct pitting patterns with other cells, both axial and radial. *Croton* is not the only taxon with such a distinction between types of procumbent cells; taxa in many families and orders bear these distinct forms of procumbent ray cells (A. C. Wiedenhoef, pers. observ.).

**20.** Perforated ray cells 0 = absent, 1 = present

These are ray cells of the same dimension or larger than the adjacent cells, but with perforations, which generally are on the side walls connecting two vessels on either side of the ray.

**21.** Disjunctive ray parenchyma cell walls 0 = absent, 1 = present

Refers to ray parenchyma cells partially disjoined but with contact maintained through tubular or complex wall processes.

**22.** Laticifers 0 = absent, 1 = present

These are cells or series of cells of indeterminate length (tubes) that extend radially, and which contain latex. The latex may be colourless, light yellow or reddish brown.

Crystals are frequently found in wood, with prismatic (rhombohedral) crystals being the most common type. They are mainly composed of calcium oxalate, which makes them birefringent under polarized light, and their shape can vary (i.e. octahedral, druses, raphides, acicular, styloids or elongate). The relative abundance of prismatic crystals is variable and they can occur in one or more cell types, throughout the ray and/or axial parenchyma, and in chambered or subdivided cells.

**23.** Crystals in upright and/or square ray cells 0 = absent, 1 = present

**24.** Crystals in chambered upright and/or square ray cells 0 = absent, 1 = present

**25.** Crystals in upright ray cells 0 = absent, 1 = present

**26.** Crystals in upright chambered ray cells 0 = absent, 1 = present

**27.** Crystals in square ray cells 0 = absent, 1 = present

**28.** Crystals in square chambered ray cells 0 = absent, 1 = present

**29.** Crystals in procumbent ray cells 0 = absent, 1 = present

**30.** Crystals in nominal procumbent ray cells 0 = absent, 1 = present

**31.** Crystals in true procumbent ray cells 0 = absent, 1 = present

**32.** More than one crystal per ray cell 0 = absent, 1 = present

**33.** Crystals in non-chambered axial parenchyma cells 0 = absent, 1 = present

**34.** Crystals in chambered axial parenchyma cells 0 = absent, 1 = present

**35.** Crystals in enlarged cells (idioblasts) 0 = absent, 1 = present

These enlarged cells can be either ray or axial parenchyma cells, and the crystals may be of any type.

**36.** Helical thickenings in the vessel elements 0 = absent, 1 = present in body and/or tails

When present, helical thickenings can be found either in the tails of the vessel elements, or throughout their body.