

Seed size and the evolution of leaf defences

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Summary

1. Leaf defences vary widely among tree species, affecting rates of herbivory, survival and reproduction.
2. Two contrasting hypotheses account for variation in leaf defences among species. The first predicts that a slow life history, which is characteristic of larger seeded species adapted to resource-limited environments, is associated with well-defended leaves. The second, apparency theory, predicts that elevated leaf defences are necessitated for species that are more detectable to herbivores.
3. Here we use comparative methods and a global data set to test (i) the relationship between seed size and leaf defences and (ii) the relationship between clumping (spatial apparency) and leaf defences.
4. We found that seed size was positively related to leaf fracture toughness, but not phenolics or tannin concentration and that spatial aggregation was unrelated to leaf defences.
5. *Synthesis.* Our results suggest that larger seed size and increased leaf toughness are correlated as part of a trait syndrome associated with a slow, resource-limited life history, not clumped dispersion and increased spatial apparency.

Key-words: fracture toughness, herbivory, plant development and life-history traits, seed dispersal, spatial aggregation

Introduction

Plant functional traits are morphological, physiological or phenological attributes that determine ecological strategies and which interact with the environment to influence the survival and reproduction of a species (Pérez-Harguindeguy *et al.* 2013). For example, seed size can influence habitat selection (Hewitt 1998), growth rate and survival (Paz & Martínez-Ramos 2003; Moles & Westoby 2006), tolerance to herbivory (Foster 1986; Harms & Dalling 1997) and mechanisms of dispersal (Tiffney 1984; Moles *et al.* 2005; Thomson *et al.* 2010) and is therefore central to the life history of many plants. Likewise, investment in the mechanical and chemical defences of leaf tissues deters herbivory and increases resistance to abiotic stresses (Coley 1983, 1987; Mithöfer & Boland 2012). The potential for natural selection to act on functional traits is high, and adaptive explanations

for variation in plant functional traits are common (Reich *et al.* 2003).

Functional traits are often involved in correlated syndromes across environmental gradients (Reich *et al.* 2003). Correlated trait syndromes can reflect strict physical relationships [i.e. increased leaf longevity requires greater tissue density for support (Kitajima *et al.* 2012)] or correlated evolutionary responses to selective pressures in a particular habitat or niche (Westoby *et al.* 2002). For example, the same factors that favour the evolution of larger seeds –i.e. high-competition, low-resource environments that limit growth and development (Foster 1986; Westoby, Jurado & Leishman 1992; Leishman & Westoby 1994a,b; Burke & Grime 1996; Westoby *et al.* 1996, 2002; Moles & Westoby 2006; Moles *et al.* 2007)– also favour life-history attributes such as shade tolerance, slow growth rates and long life spans (Coley, Bryant & Chapin 1985; Coley 1988; Coley & Barone 1996; Endara & Coley 2011; Adler *et al.* 2014). These traits, in turn, are strongly correlated with elevated leaf toughness, the primary physical

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deterrent of herbivory across the tropics (Coley 1983, 1987; Reich *et al.* 1991; Peeters, Sanson & Read 2007; Grubb *et al.* 2008; Onoda *et al.* 2011). Seed size/quality and leaf defence should therefore be correlated components of a plant life-history strategy that is ultimately driven by resource availability (Coley, Bryant & Chapin 1985) and the 'tolerance-fecundity' trade-off (Muller-Landau 2010). Variation in leaf defence across species is thus expected to vary as a function of seed size, with larger seeded species evincing greater investment in leaf defences. A direct link between seed size and leaf defence, however, has never been tested.

Plant apparency theory (Feeny 1976; Rhoades & Cates 1976) posits another (and not incompatible) mechanism to explain interspecific variation in leaf defences. Apparency theory hypothesizes that leaf defences should reflect the relative likelihood of discovery by potential herbivores. Highly apparent species, or those 'bound to be found' by herbivores, are predicted to invest more in leaf defences, particularly quantitative traits such as mechanical properties or tannins, which are often viewed as effective cumulative defences against both generalist and specialist herbivores (Feeny 1976).

Apparency is inherently a general property that encompasses a variety of plant traits and emergent properties; and, crucially, it exists as both a temporal and spatial concept: 'The susceptibility of an individual plant to discovery by its enemies may be influenced not only by its size, growth form and persistence, but also by the relative abundance of its species within the overall community' (Feeny 1976:5). Functional traits or species-level properties that increase the likelihood of detection by herbivores are therefore expected to correlate with leaf defences, although apparency is a relative measure given that the sensory systems of herbivores vary (Stanton 1983; Miller, McArthur & Smethurst 2006).

Spatial apparency could have profound effects on differential investment in leaf defences across plants. For example, species that are clumped in space are predicted to be more detectable by invertebrate and mammalian herbivores that respond proportionately to the collective size of a food target (Menzel & Backhaus 1991; Janson & Di Bitetti 1997). As such, spatially aggregated species are predicted to invest more in leaf defences than species with diffuse distributions.

Numerous tests of apparency theory have been conducted, including a recent meta-analysis that contrasted it with the major competing explanation of variation in leaf defences, the resource availability hypothesis (Endara & Coley 2011). Past studies (e.g. Coley, Bryant & Chapin 1985; Southwood, Brown & Reader 1986; Coley 1987; Silvertown & Dodd 1996), however, have focused primarily on temporal traits – growth form, life span and successional stage – as metrics of apparency. The effect of spatial apparency on the evolution of leaf defences, on the other hand, is little studied [but see Agrawal, Lau & Hambäck (2006) for a brief discussion of how plant diversity and community structure can affect apparency and Howe (1989) for a hypothesis relating dispersion of seeds and seedlings to defensive traits during those life stages].

In the most detailed existing empirical study of spatial apparency, Coley (1983) examined herbivory as a function of spatial distribution and compared patterns of damage between 'pioneer' (shade intolerant) and 'persistent' (shade tolerant) species in light gaps. Herbivory in 42 tropical tree species was independent of spatial distribution for individuals within a species and pioneer and persistent species did not differ in clumping or density within light gaps (Coley 1983). Further, an experimental test found no evidence that density influenced herbivory in three species (Coley 1983). As a result, Coley (1983) concluded that increased leaf defences observed in persistent species were unlikely to be an adaptive response to spatial apparency.

Although Coley's (1983) analyses provide evidence against spatial escape for individuals within a species, spatial distribution at the species level was only compared between classes (pioneer vs. persistent) of individuals in light gaps. As a result, continuous variation in shade tolerance and leaf defences was obscured and clumping patterns extending beyond the light gaps were not considered. Modern large-scale studies (such as Center for Tropical Forest Science plots at BCI and Pasoh) that monitor plant traits and adult distributions across large areas make it possible to dramatically increase sample size and test the role of species-level spatial apparency using the comparative method and continuous metrics of spatial aggregation and leaf defences.

Here we evaluate the hypothesized associations between seed size, spatial dispersion and leaf defence in tropical forests on three continents using two metrics of spatial aggregation and three measures of leaf defence (fracture toughness, phenols, and tannins). We test the following predictions: (i) leaf defences are positively associated with seed size as part of a suite of correlated life-history traits and (ii) leaf defences vary in relation to spatial dispersion, with more clumped (apparent) species investing more in leaf defence.

Materials and methods

STUDY LOCATIONS

Barro Colorado Island (BCI), Parque Natural Metropolitan (PNM) and San Lorenzo Protected Area (SLPA), Panama (9°9'N, 79°51'W), are lowland forests receiving ca. 2600, 1740 and 3100 mm of rainfall year⁻¹, respectively (Leigh 1999). Data on plant functional traits at BCI were collected as part of a broader study (Wright *et al.* 2010). At BCI, we collected mature leaves from the understorey or shaded conditions with pruning shears and a telescoping pole. We collected mature leaves from fully sun-exposed conditions with a shotgun and steel pellets. At PNM and SLPA, we collected mature canopy leaves with construction cranes (Parker, Smith & Hogan 1992). Plant taxonomy follows Croat (1978) and Condit, Hubbell & Foster (1995).

Bukit Timah Nature Reserve (BTNR), Singapore (1°21'N, 103°47'E), supports primary lowland dipterocarp and secondary forests and receives ca. 2600 mm of rainfall year⁻¹ (Corlett & Lucas 1990). We collected mature leaf specimens from the understorey at this site with pruning shears and a telescoping pole. Plant taxonomy follows Turner (1995). Published seed data from Bukit Timah were combined with original data (see below) from Pasoh Forest Reserve,

Malaysia (2°59'N, 102°18'E), a primary lowland dipterocarp forest receiving ca. 2000 mm of rainfall year⁻¹ (Kochummen, LaFrankie & Manokaran 1990). Plant taxonomy follows Kochummen (1997).

Kibale National Park, Uganda (0°13' – 0°41'N; 30°19' – 30°32'E), is a moist evergreen forest receiving ca. 1600 mm of rainfall year⁻¹ (Struhsaker 1997). All samples collected at this site were mature leaves adjacent to those consumed by four primate species (Dominy & Lucas 2001). We selected mature leaves to reduce the confounding effect of ontogeny on leaf defences (Boege & Marquis 2005). We obtained leaves directly from the canopy or from fallen fragments disturbed during primate foraging. Plant taxonomy follows Hamilton (1991).

PHYSICAL MEASURES

Spatial dispersion

Condit *et al.* (1999) have described the 50-ha plots located in Pasoh Forest and on BCI, Panama. At both sites, all free-standing woody stems ≥ 1 cm in diameter at breast height (dbh) were identified, tagged and mapped. The 1987 census at Pasoh recorded 815 species and 335 000 individuals. The 1982 census on BCI recorded 305 species and 235 000 individuals (Condit *et al.* 1999). Nearly all species at both sites were clumped statistically, that is, when the standardized index of relative conspecific density, Ω_x , is > 1 for all distances x (Condit *et al.* 2000). Here we use Ω_{0-10} to calculate the density of conspecifics within 10 m of a focal tree, averaged across all trees of a given species and relative to the overall density of a species. For example, if $\Omega_{0-10} = 2$, then conspecifics are twice as dense on average within a 10 m radius of a conspecific than they are across the 50-ha plot. We chose this scale because it represents the greatest degree of clumping (Condit *et al.* 2000). Ω_{0-10} was $\log_{10}+1$ -transformed when necessary to improve homoscedasticity and normality.

In addition, we utilized a second index of spatial aggregation, σ , for species at BCI and Pasoh (Plotkin *et al.* 2000; Seidler & Plotkin 2006). σ is a measure of mean cluster size, estimated by generating a Poisson cluster point process and fitting it to the observed distribution of a species in a selected area. Low values of σ signify tight spatial clustering, whereas high σ values indicate a large degree of dispersion. σ was \log_{10} -transformed to improve homoscedasticity and normality and because this variable is upper-bounded at a value of 500 m (Seidler & Plotkin 2006).

For both σ and Ω_{0-10} , species with <25 individuals used in the calculation of spatial dispersion were excluded from analyses because extreme values resulted from the low sample sizes.

Diaspore and seed size

We distinguish between diaspore size, which includes the endocarp, seed coat, endosperm and embryo that is dispersed, and seed size, which is measured by dissecting the endocarp and seed coat and measuring the remaining endosperm and embryo. We used two measures of size: diaspore volume and seed mass. Alternative measures were used at different sites because data were collected by two different research groups and combined for this study. At BCI, we used dial callipers to measure length, width and breadth, and calculated the volume of an ellipsoid as: $[(4/3)\pi(\text{length}/2)(\text{width}/2)(\text{breadth}/2)]$. We measured mass with a digital scale before and after drying to obtain fresh and dry mass, respectively. The present analyses are based on seed dry mass as it reflects the nutritional investment in offspring. For Kibale species, we obtained diaspore dimensions from Zanne,

Chapman & Kitajima (2005) and the unpublished records of R.S. Duncan (Birmingham Southern College). For Pasoh species, we obtained diaspore dimensions from Corlett & Lucas (1990) and Lucas & Corlett (1998), who worked in Bukit Timah, Singapore. Additional data were collected in Pasoh during a low-intensity general masting event in 2002 (Numata *et al.* 2003). Seed mass and diaspore volume were \log_{10} -transformed to improve the normality of residuals, homoscedasticity and linearity in regressions.

Leaf toughness

Toughness is frequently measured with a penetrometer, a device that forces a circular flattened rod through leaf lamina. However, penetrometers do not measure fracture toughness or any other fundamental mechanical property (Vincent 1990; Choong *et al.* 1992; Aranwela, Sanson & Read 1999). Here we regard fracture toughness, or material resistance to crack propagation, as biologically important because it is the central property responsible for maintaining the structural integrity of an organism. It is defined as the energy consumed in growing a crack of given area and, in leaves, it plays a critical role in resisting pathogens, herbivores and other physical damage (Choong *et al.* 1992; Lucas *et al.* 2000; Dominy *et al.* 2008). We performed separate analyses for canopy and understorey leaves when possible because substantial differences have been previously demonstrated between leaves growing in different light regimes (Dominy, Lucas & Wright 2003; Onoda *et al.* 2011) and because we observed large differences in leaf toughness between those groups. For the same reason, we performed separate analyses for sun-exposed and shade leaves at BCI.

Leaf fracture toughness was measured with a pair of scissors (Dovo, Germany) to control and direct crack growth (Lucas & Pereira 1990). The scissors, with an included angle of 55° and a radius of curvature (sharpness) of 1.6 μm , were mounted on a portable universal testing machine (Darvell *et al.* 1996). We fractured leaves in a transverse cut perpendicular to the midrib (Fig. 1). This method, described by Lucas *et al.* (2001), allows the toughness (in J m^{-2}) of individual anatomical features, such as the secondary veins and lamina, to be calculated from a single scissors pass. In Kibale, a leaf was subjected to five cuts (#2 – #6) from the base to the apex (Fig. 1), the mean of which is reported here. In subsequent work at PNM, SLPA and Bukit Timah, we calculated cut #4 in a sample of four mature leaves due to the strong correlation between it and the average value of cuts #2–6 at Kibale ($n = 149$ species, $r = 0.85$, $P < 0.001$). Multiple leaf samples were generally collected from a single plant. On BCI, we collected three leaf samples from each of ~ 5 individuals per specie and made a single cut near position #3 (Fig. 1). Leaf toughness was \log_{10} -transformed prior to analysis.

We also measured leaf dimensions in order to test for allometric effects. We measured maximum leaf width with dial callipers and leaf thickness with a micrometer (SM112; Teclock, Okayashi, Japan). We measured leaf thickness of the lamina halfway between the midrib and the leaf margin at cut position #4 (Fig. 1). Both variables were \log_{10} -transformed prior to analysis.

CHEMICAL MEASURES

Chemical extraction

Waterman & Mole (1994) discuss extraction techniques for quantifying phenolic compounds, including a review of the various solvents possible. From this discussion and a consideration of tests for other

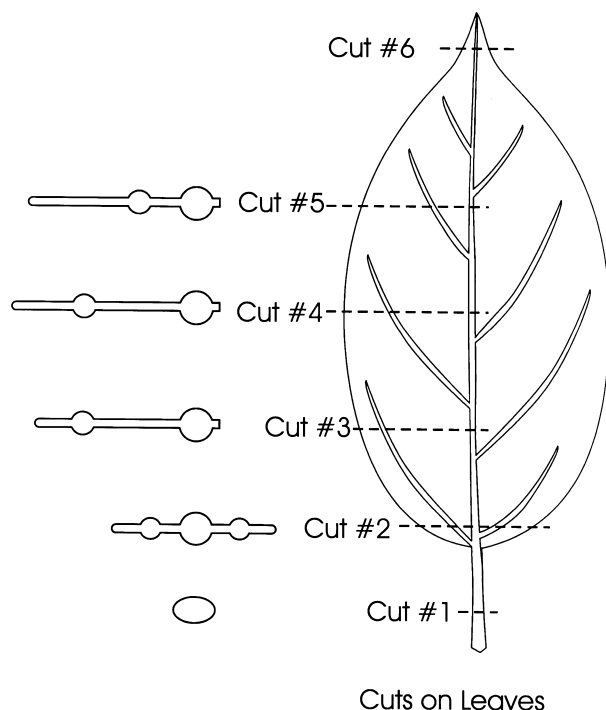


Fig. 1. A typical leaf and the arrangement of cuts used to study fracture toughness. Work (J , for Joules) is divided by cross-sectional area to yield fracture toughness ($J\ m^{-2}$). The cross-sectional areas assumed from a cut are shown to the left of the leaf. Following Choong (1996), laminae were treated as rectangular and secondary veins treated as circular.

constituents, 50% methanol (1:1 $dH_2O:CH_3OH$) was chosen as the solvent. Approximately 0.1 g of fresh plant tissue was weighed, cut into approximately 1-mm pieces and extracted in 5-mL of 50% methanol with a tissue homogenizer (Tissue Tearor; Dremel, Racine, WI, USA). We then collected the homogenate into a 10-mL syringe fitted with a Luer lock and fibreglass filter (1.6 μm pore size, type 1; Millipore, Billerica, MA, USA). Slow depression of the syringe plunger forced the homogenate through the filter and into a 1.5-mL Eppendorf tube, where samples were stored for the analyses described below (Lucas *et al.* 2001). Note that these tests utilize fresh rather than dried material, allowing results to be expressed as concentrations on a fresh mass basis (as sensed by an herbivore) rather than on a dry weight basis (which relates to nutritional gain).

Phenolics

Concentrations of total phenolic compounds were measured by the Prussian blue test (Price & Butler 1977) as modified by Graham (1992), Hagerman (1998) and Lucas *et al.* (2001). Results are expressed as percentage equivalents to a 4-point standard curve of gallic acid (Aldrich, St. Louis, MO, USA). Appel *et al.* (2001) note that this method measures the reducing capacity of phenols, not necessarily the overall concentration. In some cases, this variable was \log_{10} -transformed to meet assumptions of normality.

Tannins

We quantified tannins following the method of Hagerman (1987) and modifications of Lucas *et al.* (2001). We loaded plant extracts into

the pre-moulded wells of a BSA-laden, agarose gel (type I; low EEO; Sigma, St. Louis, MO, USA), where tannin precipitation produces visible rings. We then measured ring dimensions with dial callipers, and results are expressed as percentage equivalents to an 8-point standard curve of crude quebracho tannin (gift of Professor A.E. Hagerman, Miami University, Ohio). In some cases, this variable was \log_{10} -transformed to meet assumptions of normality.

STATISTICAL ANALYSES

Phylogeny construction

Closely related taxa are expected to have similar trait values and therefore violate assumptions of independence for traditional statistical analysis. To account for the potentially confounding effects of phylogenetic relatedness in comparative analysis, we obtained a phylogenetic tree for each data set and used a phylogenetic generalized least squares (PGLS) approach. For species at BCI, we used an updated version of the dated DNA sequence-derived barcode phylogeny from Kress *et al.* (2009). In a small number of cases (maximum six species for a given analysis), species pairs in the phylogeny were separated by zero-length terminal branches. In those cases, one species in each pair was randomly selected and removed from the analysis. All applicable analyses were also replicated with different combinations of species removed to ensure that results were insensitive to the removals. At all other sites, we estimated phylogenetic relationships between species using the Phylomatic module in PHYLOCOM 4.1 (Webb, Ackerly & Kembel 2008). Phylomatic attaches an input list of taxa to a known megatree template; here, we used tree version R20091110 (Bremer *et al.* 2009). Due to a lack of complete resolution, unrecognized species and genera were attached to polytomous genus and family nodes, respectively. To ensure taxonomic agreement with the megatree template, the original taxonomies were modified using synonyms from the International Plant Names Index (<http://www.ipni.org>). Finally, branch lengths were added to phylogenies using the Bladj function in Phylocom, in which dated nodes from Wikström, Savolainen & Chase (2001) were assigned fixed ages with remaining nodes placed evenly in between them. All phylogeny files used in this study are available upon request.

Phylogenetic generalized least squares (PGLS)

We performed all statistical analyses in R 3.0.2 (R Core Team 2013). For PGLS, we used the packages caper (Orme *et al.* 2013) and ape (Paradis, Claude & Strimmer 2004) designed for comparative analysis of phylogenetic data. Whereas components of the error term in an ordinary least squares regression are assumed to follow a normal distribution around a mean of zero and variance σ^2 , PGLS corrects for the effect of non-independence in observations by incorporating phylogeny and an expected model of evolution into the variance-covariance matrix specified for the error term of a linear model (Martins & Hansen 1997; Garland & Ives 2000).

Our analyses assumed a simple continuous evolutionary model of Brownian motion subject to the scaling factor, λ (Pagel 1999). λ , estimated in each model using a maximum likelihood (ML) approach, is a constant that allows one to assess the strength of a phylogenetic signal in the residuals of a regression model. Specifically, a value of $\lambda = 0$ indicates phylogenetic independence among observations [and thus the resulting PGLS is equivalent to ordinary least squares (OLS)], while a value of $\lambda = 1$ indicates that species traits covary directly according to a Brownian motion model of evolution (Freckleton, Harvey & Pagel

2002). PGLS was preferred for phylogenetic linear regression analyses because current software supports the estimation of λ using maximum likelihood, as opposed to independent contrasts, which assume strict Brownian motion ($\lambda = 1$). Following each PGLS analysis, we generated diagnostic plots to assess standard assumptions of linear models after adjusting for phylogeny (Freckleton 2009).

For the PGLS analysis, we paired leaf fracture toughness data from PNM and SLPA with diaspore volume data drawn from the collections at BCI. This approach reflects the presence of canopy cranes at PNM and SLPA and the opportunity to collect leaves directly from the canopy. Similarly, leaf toughness values at Bukit Timah were integrated with measures of Ω and σ from Pasoh.

Due to the large number of hypothesis tests performed, we used the Benjamini–Hochberg method to control the false discovery rate within each general family of tests and we present both corrected and uncorrected P values for all relevant analyses (Benjamini & Hochberg 1995). All results are interpreted using B-H corrected P values.

Phylogenetic signal

We tested individual traits for phylogenetic signal using two frequently used metrics, Pagel's λ (Pagel 1999) and Blomberg's K (Blomberg, Garland & Ives 2003). To do so, we used the `phylosig` function in the `phytools` package in `R` (Revell 2012). Both Pagel's λ and Blomberg's K assess the degree to which a trait exhibits phylogenetic signal according to a Brownian motion model of evolution. We used a randomization test with 10 000 simulations to test for significance in phylogenetic signal.

Results

SEED SIZE AND LEAF DEFENCE

Leaf lamina fracture toughness was positively and significantly related to seed volume at all sites (Table 1; Fig. 2). Similarly, the fracture toughness of secondary veins in canopy

and understorey leaves was positively related to seed volume at sites in Singapore and Panama (Table 1; Fig. 2). Although leaf secondary vein fracture toughness was not significantly related to seed volume at Kibale, Uganda (Table 1; B-H adjusted $P = 0.204$), the trend was consistent with those from other sites. Leaf lamina and secondary vein fracture toughness were also positively and significantly related to dry seed mass at BCI for both sun and shade leaves (Table 1; Fig. 3). Lastly, despite substantial variation in the concentrations of chemical defences, seed volume was a poor predictor of the concentrations of phenolics and tannins in mature leaves (Table 2; B-H adjusted $P > 0.05$ for all sites).

We also analysed the relationship between leaf size and fracture toughness given the potential allometry between seed size, plant size and leaf size (Thompson & Rabinowitz 1989; Kelly 1995; Cornelissen 1999; Mizukami & Fischer 2000) and the possibility that larger leaves are tougher simply to provide architectural support (Wright & Cannon 2001). Leaf width is the most reliable indicator of leaf size (Turner 2001), and leaf width was unrelated to leaf lamina and secondary vein toughness at any site (Table S1 in Supporting information). Leaf thickness was a similarly poor predictor of leaf fracture toughness (Westbrook *et al.* 2011; Kitajima *et al.* 2012) and was not significantly correlated with lamina or secondary vein fracture toughness at any site (Table S1 in Supporting information).

SPATIAL DISPERSION AND LEAF DEFENCE

Two metrics of spatial dispersion, Ω_{0-10} and σ , were used to predict leaf defences. After accounting for the potential effects of phylogeny, we found that lamina and secondary vein toughness were unrelated to spatial dispersion (Ω_{0-10} and σ) at all sites (Table 3; Fig. 4). For chemical defences at PNM

Table 1. Phylogenetic generalized least squares analysis of the relationship between diaspore volume or seed dry mass and leaf fracture toughness (lamina and secondary vein)

Region: Site (leaf location)	β (SE)	P	λ^\dagger	n	B-H adjusted P
Lamina toughness ~ Diaspore volume					
Panama: PNM and SLPA (understorey)	0.067 (0.023)	0.005	0.000	41	$6.6 \times 10^{-3} **$
Panama: PNM and SLPA (canopy)	0.084 (0.027)	4.6×10^{-3}	0.000	30	$6.6 \times 10^{-3} **$
Singapore: Bukit Timah (understorey)	0.037 (0.010)	0.001	0.000	49	$2.0 \times 10^{-3} **$
Uganda: Kibale (canopy)	0.099 (0.027)	6.5×10^{-4}	0.134	46	$2.0 \times 10^{-3} **$
Secondary vein toughness ~ Diaspore volume					
Panama: PNM and SLPA (understorey)	0.104 (0.029)	0.001	0.000	34	$2.0 \times 10^{-3} **$
Panama: PNM and SLPA (canopy)	0.080 (0.028)	0.008	0.474	25	$9.6 \times 10^{-3} **$
Singapore: Bukit Timah (understorey)	0.063 (0.012)	7.9×10^{-6}	0.000	43	$4.7 \times 10^{-5} **$
Uganda: Kibale (canopy)	0.063 (0.049)	0.204	0.504	35	0.204
Lamina toughness ~ Seed mass					
Panama: BCI (shade)	0.069 (0.013)	5.4×10^{-7}	0.148	172	$6.5 \times 10^{-6} **$
Panama: BCI (sun)	0.046 (0.015)	2.5×10^{-3}	0.167	119	$4.3 \times 10^{-3} **$
Secondary vein toughness ~ Seed mass					
Panama: BCI (shade)	0.055 (0.013)	2.8×10^{-5}	0.283	172	$1.1 \times 10^{-4} **$
Panama: BCI (sun)	0.033 (0.013)	0.013	0.349	119	0.014 **

*B-H adjusted $P < 0.05$, **B-H adjusted $P < 0.01$.

\dagger The parameter λ [ML] is a measure of phylogenetic signal included as a parameter in the models, shown here as a maximum likelihood estimate.

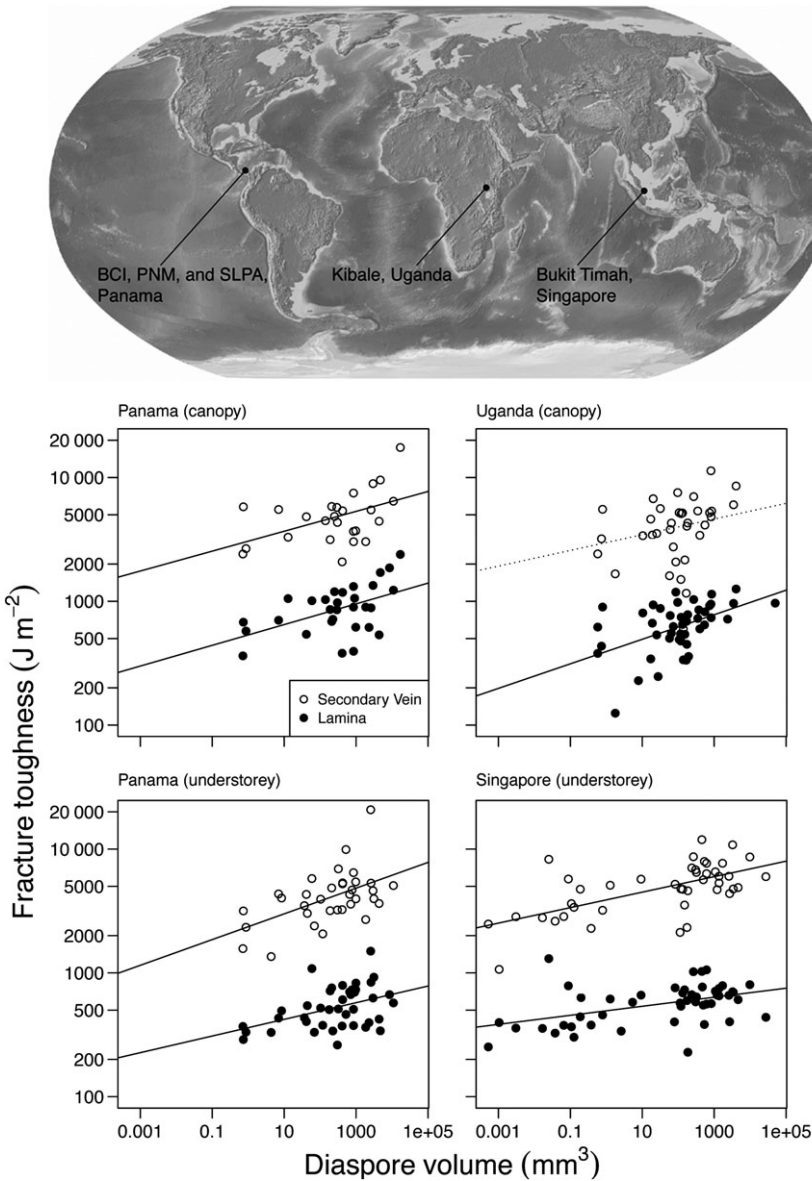


Fig. 2. Relationship between lamina (solid circles) or secondary vein (open circles) fracture toughness and diaspore volume. In Panama, diaspore volumes were obtained using the archives at BCI, and leaf fracture toughness values were obtained using canopy cranes at PNM and SLPA. Regression lines are from PGLS analyses. Solid lines represent significant relationships ($P < 0.05$) and dashed lines represent non-significant relationships. Points represent raw mean values for individual species.

and SLPA, the concentration of leaf tannins was unrelated to spatial dispersion in both canopy and understorey leaves (Table 2). The concentration of leaf phenolics was negatively related to σ in understorey leaves, but was otherwise unrelated to spatial dispersion (Table 2).

PHYLOGENETIC SIGNAL

Measures of phylogenetic signal (measured as Pagel's λ and Blomberg's K) and summary statistics for individual traits are presented in Table S2 (Supporting information). We detected significant phylogenetic signal in 7 out of 26 traits across sites for Pagel's λ and in 12 out of 26 traits across sites for Blomberg's K (Table S2 in Supporting information). Traits measured at BCI tended to have the highest phylogenetic conservatism (4/7 and 5/7 traits with significant phylogenetic signal for Pagel's λ and Blomberg's K , respectively), possibly

due to the use of a high-resolution barcode phylogeny at this site.

Discussion

Numerous factors have been proposed to explain variation in plant defences across species (Agrawal 2007). Using a global data set, we investigated two hypotheses to explain leaf defence variation based on previous empirical and theoretical work. The first hypothesis, which reflects life-history trade-offs between seed size/quality and number of seeds produced, treats seed size and leaf defence as correlated components of life-history strategy. Experimental evidence indicates that the seedlings of larger seeded species are more tolerant of numerous hazards, including competition, shade, defoliation, nutrient shortage, litter cover and drought (reviewed in Westoby *et al.* 1996; Moles & Westoby 2004). In turn, larger seeded plants

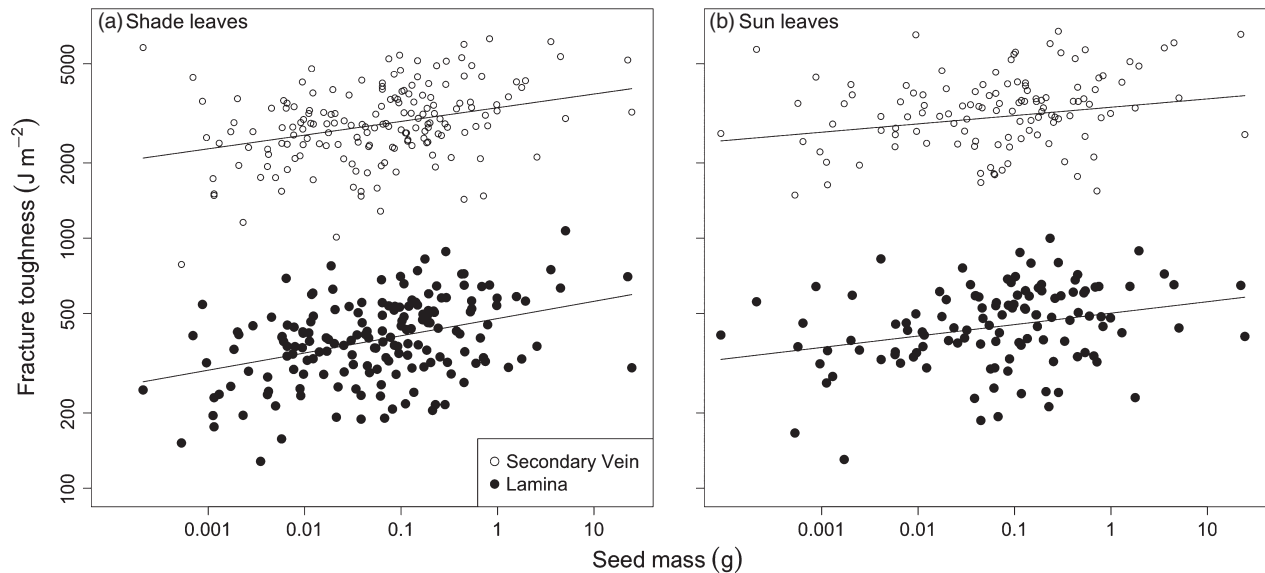


Fig. 3. Relationship between (a) shade and (b) sun lamina (solid circles) or secondary vein (open circles) fracture toughness and dry seed mass at BCI (Panama). Regression lines are from PGLS analyses. Points represent means for individual species.

exhibit multiple life-history traits that support tolerance of low resources, including slow growth rate (Swanborough & Westoby 1996) and long leaf lifetime (Westoby *et al.* 2002), that are correlated with increased leaf defences and decreased herbivory (Coley 1988; Coley & Barone 1996; Endara & Coley 2011). Investments in seed size/quality and leaf defence are therefore predicted to increase in a coordinated manner that is ultimately dependent on environmental conditions and resource availability (Coley, Bryant & Chapin 1985).

A second hypothesis, based on apparency theory (Feeny 1976), posits that spatially aggregated species are more easily detected and are therefore more vulnerable to herbivory pressure. As a result, spatially clumped species are predicted to invest more in leaf defences, particularly quantitative traits such as leaf toughness (Feeny 1976). Similarly, Howe (1989) proposed spatial apparency as the basis for the ‘scatter-clump’ hypothesis, which predicts an indirect correlation between seed size and defence mediated by spatial aggregation. The saplings

Table 2. PGLS analysis of the relationship between diaspore volume or spatial dispersion and leaf phenolic or tannin concentration

Region: site (leaf location)	β (SE)	P	λ^\dagger	n	B-H adjusted P
Diaspore volume: Phenolic concentration ~ Diaspore volume					
Panama: PNM and SLPA (understorey)	0.102 (0.124)	0.417	0.000	36	0.804 [‡]
Panama: PNM and SLPA (canopy)	0.176 (0.281)	0.536	0.000	30	0.804 [‡]
Uganda: Kibale (canopy)	0.010 (0.157)	0.952	0.000	41	0.970 [‡]
Tannin concentration ~ Diaspore volume					
Panama: PNM and SLPA (understorey)	0.223 (0.277)	0.426	0.000	36	0.804 [‡]
Panama: PNM and SLPA (canopy)	0.386 (0.601)	0.526	0.000	30	0.804 [‡]
Uganda: Kibale (canopy)	-0.027 (0.728)	0.970	0.000	41	0.970 [‡]
Spatial dispersion:					
Phenolic concentration ~ Ω					
Panama: PNM and SLPA (understorey)	0.152 (0.249)	0.547	0.000	31	0.729 [§]
Panama: PNM and SLPA (canopy)	0.374 (0.151)	0.021	0.000	25	0.084 [§]
Tannin concentration ~ Ω					
Panama: PNM and SLPA (understorey)	0.203 (0.874)	0.818	0.000	31	0.818 [§]
Panama: PNM and SLPA (canopy)	1.516 (1.842)	0.419	0.000	25	0.678 [§]
Phenolic concentration ~ σ					
Panama: PNM and SLPA (understorey)	-0.481 (0.144)	0.002	0.573	30	0.016 ^{§,*}
Panama: PNM and SLPA (canopy)	-0.148 (0.146)	0.322	0.000	23	0.678 [§]
Tannin concentration ~ σ					
Panama: PNM and SLPA (understorey)	0.519 (0.639)	0.424	0.000	30	0.678 [§]
Panama: PNM and SLPA (canopy)	-0.764 (1.668)	0.651	0.000	23	0.744 [§]

*B-H adjusted $P < 0.05$.

[†]The parameter λ [ML] is a measure of phylogenetic signal included as a parameter in the models, shown here as a maximum likelihood estimate.

^{‡,§}Matching superscripts indicate the family of tests included in the Benjamini–Hochberg adjustment.

Table 3. Phylogenetic generalized least squares analysis of the relationship between spatial dispersion (Ω or σ) and leaf fracture toughness (lamina and secondary vein)

Region: site (leaf location)	β (SE)	P	λ^*	n	B-H adjusted P
Lamina toughness $\sim \Omega$					
Panama: PNM and SLPA (understorey)	-0.074 (0.071)	0.302	0.000	34	0.571
Panama: PNM and SLPA (canopy)	-0.093 (0.091)	0.314	0.000	25	0.571
Singapore: Bukit Timah (understorey)	-0.028 (0.091)	0.764	0.000	21	0.840
Panama: BCI (shade)	-0.033 (0.029)	0.255	0.451	158	0.567
Panama: BCI (sun)	-0.055 (0.041)	0.187	0.369	111	0.533
Secondary vein toughness $\sim \Omega$					
Panama: PNM and SLPA (understorey)	-0.153 (0.075)	0.050	0.771	30	0.480
Panama: PNM and SLPA (canopy)	-0.114 (0.089)	0.213	0.726	21	0.533
Singapore: Bukit Timah (understorey)	0.027 (0.105)	0.798	1.000	18	0.840
Panama: BCI (shade)	-0.038 (0.025)	0.138	0.549	158	0.480
Panama: BCI (sun)	-0.020 (0.035)	0.569	0.450	111	0.759
Lamina toughness $\sim \sigma$					
Panama: PNM and SLPA (understorey)	0.004 (0.063)	0.945	0.000	33	0.945
Panama: PNM and SLPA (canopy)	-0.025 (0.088)	0.779	0.000	23	0.840
Singapore: Bukit Timah (understorey)	0.108 (0.070)	0.144	0.000	17	0.480
Panama: BCI (shade)	0.041 (0.027)	0.138	0.442	180	0.480
Panama: BCI (sun)	0.014 (0.035)	0.407	0.356	127	0.581
Secondary vein toughness $\sim \sigma$					
Panama: PNM and SLPA (understorey)	-0.068 (0.077)	0.385	0.728	29	0.581
Panama: PNM and SLPA (canopy)	0.039 (0.094)	0.684	0.740	19	0.840
Panama: Bukit Timah (understorey)	0.104 (0.119)	0.395	1.000	16	0.581
Panama: BCI (shade)	0.046 (0.023)	0.043	0.646	180	0.480
Panama: BCI (sun)	0.048 (0.028)	0.089	0.566	127	0.480

*The parameter λ [ML] is a measure of phylogenetic signal included as a parameter in the models, shown here as a maximum likelihood estimate.

and seeds of larger seeded species are predicted to be highly aggregated due to dispersal by large organisms that deposit seeds in clumps, increasing apparency to herbivores and necessitating a greater investment in defences for early life stages.

Plant species that are spatially aggregated or which exist in high density may be more easily detected by herbivores using visual or chemical cues (Stanton 1983; Janson & Di Bitetti 1997). For example, it is well known that many herbivorous insects rely on visual cues to aid in the detection of host plants (Prokopy & Owens 1983), and there is evidence that the obstruction of visual cues can reduce the probability of detection (Rausher 1981). Likewise, insect herbivores which respond to olfactory stimuli have been observed to locate dense host plant stands more easily than sparse stands (Ralph 1977a,b; Stanton 1983).

In this study, we first tested the prediction that seed size and leaf defence are positively correlated using data collected from three continents. Diaspore volume was positively and significantly related to leaf lamina toughness at all sites and with secondary vein toughness at two of three sites (Tables 1 and 3). Similarly, dry seed mass at BCI was positively and significantly related to leaf lamina and vein toughness for shade and sun leaves (Table 1; Fig. 4). The association between seed size and leaf defence does not appear to be an artefact of a confounding relationship between leaf size and toughness (Table S1 in Supporting information), as expected based on a previous investigation that found leaf fracture toughness to be independent of leaf thickness (Kitajima &

Poorter 2010). In contrast to toughness, diaspore volume was unrelated to the concentration of leaf tannins and phenols at all sites (Table 2). In general, results do not appear to differ between understorey and canopy or between sun and shade leaves. These findings provide supporting evidence of correlated evolution between seed size and mechanical, but not chemical, leaf defence in tropical trees.

Next, we evaluated the relationship between spatial aggregation and leaf defence as proposed by apparency theory. Although spatial dispersion is predicted to influence investment in leaf defence (Feeny 1976), we found that two measures of aggregation, Ω_{0-10} and σ , were generally unrelated to leaf fracture toughness, tannin concentration or phenol concentration of adult trees across sites (Tables 2 and 3; Fig. 2).

Several factors could explain the lack of relationship between spatial aggregation and leaf defence. First, apparency may not operate at the population or species level. Spatial apparency may be better characterized for individuals within a population, whose functional traits, such as leaf defences, can be influenced locally by phenotypic plasticity in response to herbivory or other factors (Hoy, Head & Hall 1998; Agrawal 2001; Agrawal *et al.* 2002). Consider a case in which a species is scattered diffusely in some places but is distributed in dense clusters elsewhere. Although this species will on average have a moderate value of spatial aggregation, individuals will have highly variable levels of apparency. Thus, fine-scale analyses of spatial aggregation and leaf defences for individuals within a population may be necessary, although previous

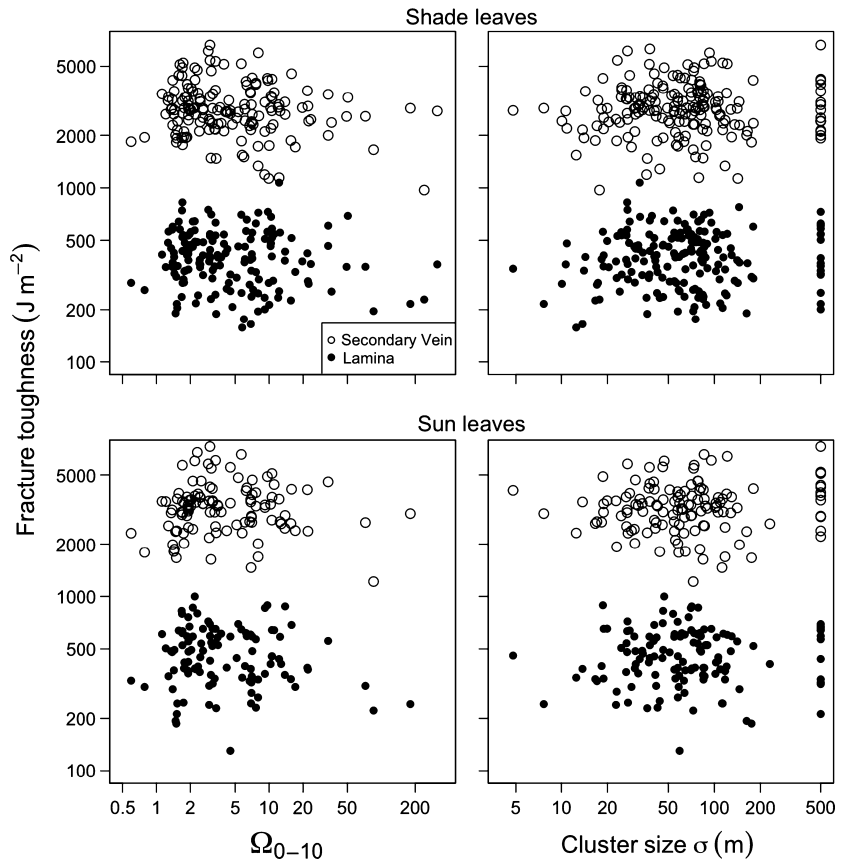


Fig. 4. Lamina (solid symbols) and secondary vein (open symbols) fracture toughness as a function of two measures of spatial aggregation at BCI (Panama). The top and bottom rows show shade and sun leaves, respectively. Lamina and vein fracture toughness were not significantly related to Ω_{0-10} or σ in any of the PGLS analyses. Ω_{0-10} was $\log_{10}(x+1)$ -transformed prior to the analysis, and two species with $\Omega_{0-10} = 0$ are not shown due to the logarithmic scale.

evidence suggests that clumping and density do not necessarily affect herbivory pressure at the individual level for some tropical species (Coley 1983).

Second, spatial aggregation as observed by potential herbivores might depend on community structure (Agrawal, Lau & Hambäck 2006). It is well known that associational resistance and susceptibility can operate in plant communities to increase or decrease herbivory pressure on heterospecifics (Barbosa *et al.* 2009). Further, herbivory damage has been found to be greater for species that are more closely related to the surrounding community (Ness, Rollinson & Whitney 2011). On the other hand, Lebrija-Trejos *et al.* (2014) found that seedling survival was improved near phylogenetically similar heterospecific neighbours. For this reason, an improved metric of spatial apparency would account for community composition and distribution as well as the dietary breadth and host preferences of potential herbivores. For example, the phylogenetic relatedness of neighbouring plants in proximity to a host could be quantified and partitioned according to divergence times (Lebrija-Trejos *et al.* 2014), and then be incorporated into dispersion metrics. As a first step, the same procedure used to define σ could be modified to measure aggregation at the genus or family level under the assumption that closely related species are likely to attract similar herbivores (Seidler & Plotkin 2006).

Third, spatial distribution may have a negligible effect on herbivory pressure relative to life-history traits (Coley 1983).

As a result, species that are more clumped on average may not have historically been under selective pressure to evolve increased leaf defences, and other factors including resource availability, life history and/or phylogenetic constraint might account for the large variation in leaf defences observed in nature.

Taken together, our results indicate that larger seed size and greater leaf fracture toughness, but not increased chemical defence, fall within a suite of life-history traits associated with greater investment per offspring. This suite includes tolerance of competition and low-resource availability, long leaf lifetime, late age of reproductive maturity, and relatively slow growth rates, factors which collectively depend on low losses to pests to ensure reproductive success. Thus, given that leaf toughness is a general defence strategy that protects against a wide variety of hazards (Turner 1994; Lucas *et al.* 2000), including insect and vertebrate herbivores (e.g. Choong 1996; Teaford *et al.* 2006; Grubb *et al.* 2008), it is not surprising that species with larger seeds also have tougher leaves.

It is less clear, however, why species with larger seeds did not also show increased concentrations of chemical defences. One possibility is that such a relationship between chemical defences and seed size exists, but for total investment in all types of chemical defences. Further, these results might be explained by the fact that in some cases, chemical defences are not significantly correlated with herbivory (Coley 1983) or key life-history parameters such as leaf life span (Kitajima *et al.* 2012). Chemical defence may therefore fall outside the

suite of correlated life-history traits associated with low-resource availability and slow growth rate. Finally, any relationship between seed size and chemical defences is likely to be obscured by our focus on mature leaves. Young leaves cannot be tough because hard cell walls preclude growth. As a result, general patterns suggest that chemical defences increase primarily during the seedling phase of development (Barton & Koricheva 2010) and a majority of existing studies have observed a negative association between plant age and chemical defence (Boege & Marquis 2005). A positive relationship between seed size and chemical defence investment is therefore most likely among trees in early life stages, highlighting the need for ontogeny to be considered in future studies.

Tropical tree species have a wide range of chemical and mechanical defences, the cost of which can be great (Strauss *et al.* 2002). Increased leaf toughness confers fitness benefits to plants (Marquis 1984) by reducing herbivory (Endara & Coley 2011) and tree and shrub mortality (Westbrook *et al.* 2011). As a result, the resources that a plant invests in leaf defence should vary according to a number of factors, including resource availability and exposure to herbivores (Feeny 1976; Coley, Bryant & Chapin 1985). Yet we found no evidence that spatial apparency has exerted a selective pressure on the leaf defences of tropical trees. Our results support the hypothesis that large seed size and high leaf fracture toughness covary as central elements of a slow, low-resource life-history strategy of tropical trees.

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Data accessibility

Data associated with this manuscript have been archived in the Dryad Digital Repository <http://dx.doi.org/105061/dryad.69ph0> (Kraft *et al.* 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. PGLS analysis of the relationship between leaf fracture toughness (lamina or secondary vein) and leaf physical attributes (leaf width or leaf thickness).

Table S2. Phylogenetic signal (Pagel's λ and Blomberg's K and associated *P* values from a randomization test) and summary statistics for measured traits.