Genome size is a strong predictor of cell size and stomatal density in angiosperms

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Summary

• Across eukaryotes phenotypic correlations with genome size are thought to scale from genome size effects on cell size. However, for plants the genome/cell size link has only been thoroughly documented within ploidy series and small subsets of herbaceous species.

• Here, the first large-scale comparative analysis is made of the relationship between genome size and cell size across 101 species of angiosperms of varying growth forms. Guard cell length and epidermal cell area were used as two metrics of cell size and, in addition, stomatal density was measured.

• There was a significant positive relationship between genome size and both guard cell length and epidermal cell area and a negative relationship with stomatal density. Independent contrast analyses revealed that these traits are undergoing correlated evolution with genome size. However, the relationship was growth form dependent (nonsignificant results within trees/shrubs), although trees had the smallest genome/cell sizes and the highest stomatal density.

• These results confirm the generality of the genome size/cell size relationship. The results also suggest that changes in genome size, with concomitant influences on stomatal size and density, may influence physiology, and perhaps play an important genetic role in determining the ecological and life-history strategy of a species.

Key words: cell size, genome size, independent contrasts, stomata, stomatal density.

Introduction

Eukaryotic genome size (nuclear DNA amount) ranges nearly five orders of magnitude. Early observations of genome size variation noted various correlations at the cellular level, including a positive correlation with nuclear volume (Baetcke *et al.*, 1967; Jovtchev *et al.*, 2006) and cell volume (Mirsky & Ris, 1951; Commoner, 1964; Darlington, 1965; Bennett, 1972; Price *et al.*, 1973), and a negative correlation with the duration of the cell cycle (Van't Hof & Sparrow, 1963; Evans *et al.*, 1972; Van't Hof, 1974). For the genome size/cell size relationship, a broad sampling of the animal kingdom has consistently reported a strong positive relationship (i.e. Horner & Macgregor, 1983; Hardie & Hebert, 2003; Organ *et al.*, 2007). For plants, many studies have relied on within-species comparisons across varying ploidy series (i.e. Mowforth & Grime, 1989; Melaragno *et al.*, 1993; Kudo & Kimura, 2002). From these studies it is apparent that polyploid cells are significantly larger than their diploid progenitors. However, comparisons across large taxonomically diverse species assemblages are sparse and the results reported in the literature are not consistent, with correlations ranging from 1.0 (Price *et al.*, 1973) to −0.48 (Grime *et al.*, 1997). Moreover, all studies of the plant genome size/cell size relationship have been carried out using limited samples of herbaceous angiosperm species. Nevertheless, the relationship between genome size and cell size is often casually assumed for plants and serves as the basis for testing genome size-dependent variation in higher phenotypic scales (Bennett, 1972, 1987; Knight *et al.*, 2005; Beaulieu *et al.*, 2007a,b). This paper examines to what extent the relationship

between genome size and cell size can be generalized across angiosperms.

Recent large-scale studies in which the genome size/cell size relationship has been assumed to influence the phenotype have often produced weak results. For example, variation in leaf mass per unit area (LMA) is correlated with cell size (Castro-Díez *et al.*, 2000). Thus, through cell size it was hypothesized that genome size could possibly be a genetic driver for LMA. However, across 274 species the relationship between genome size and LMA was weak and mostly driven by divergences of basal taxonomic groups (Beaulieu *et al.*, 2007a). Furthermore, it had long been suggested that there was a strong positive relationship between genome size and seed mass (i.e. Bennett, 1972; Thompson, 1990; Grime *et al.*, 1997; Knight & Ackerly, 2002). It was assumed that genome size was related to seed mass through cell size effects within seed organs (the endosperm, cotyledons, hyocotyls, etc). After conducting an analysis across 1222 species, Beaulieu *et al.* (2007b) discovered that this relationship was not nearly as strong as would have been predicted based on a synthesis of the primary literature, and was nuanced by threshold effects, rather than conforming to a simple linear predictive model. These results have left lingering doubts about the genome size/cell size assumption: either the relationship between genome size and cell size is not as strong as previously thought or compensatory mutations buffer other phenotypic traits, which break down genome size scaling effects (see Otto, 2007; Knight & Beaulieu, 2008). To differentiate between these possibilities we tested the genome size/cell size relationship using a broad sample of angiosperm species spanning several plant functional groups.

The positive results typically reported for the plant genome size/cell size relationship could be a consequence of not including the evolutionary history of species. Evolutionary history can result in trait similarity (termed 'phylogenetic signal') among related species and thus species values cannot be considered independent data points (Felsenstein, 1985). In addition, large divergences between deep taxonomic divisions (e.g. monocots and eudicots) can create trait correlations among extant species even though subsequent evolutionary divergences do not necessarily follow the same trend (i.e. Ackerly & Donoghue, 1998; Ackerly & Reich, 1999; Moles *et al.*, 2005; Beaulieu *et al.*, 2007a,b). For these reasons, cross-species analyses should take phylogenetic relatedness into account. Recently, the first phylogenetically informed analyses of the genome size/cell size relationship were reported for vertebrates (Organ *et al.*, 2007) and diatoms (Connolly *et al.*, 2008), confirming the positive relationship. To our knowledge no phylogenetic analyses of the genome size/cell size relationship has been carried out within higher plants.

In this study we re-examined the relationship between genome size and cell size using a comparative approach across a broad taxonomic assemblage of 101 angiosperm species of varying growth forms. We used leaf cell traits (guard cell length and epidermal cell area) as proxies for cell size and measured stomatal density using leaf impressions. We discuss potential downstream consequences of this relationship including effects on carbon gain and water use efficiency (Cowen, 1986; Raven, 2002). We examined the data in several different ways. First, we analyzed the relationship between genome size and guard cell length, epidermal cell area, and stomatal density using regression without considering phylogeny. These relationships were also analyzed for monocots, eudicots, and each growth form (herbs, shrubs, and trees), separately. Secondly, we assessed the role of phylogeny influencing trait distributions by testing for significant phylogenetic signal (Blomberg & Garland, 2002; Blomberg *et al.*, 2003). Significant phylogenetic signal indicates trait similarity among closely related species, and its presence demonstrates that the data do not satisfy the statistical assumption of independence (Felsenstein, 1985). Thirdly, we tested trait associations using independent contrasts to determine the strength of the relationships after incorporating phylogenetic information. Finally, we also incorporated phylogeny to test whether significant trait differences exhibited between monocots and eudicots, and among growth forms, could have arisen by chance.

Materials and Methods

Genome size and species selection

Guard cell length, stomatal density, and epidermal cell area were measured for 101 angiosperm species with known 2C DNA estimates growing at the Royal Botanic Gardens, Kew (RBG, Kew, UK). Estimates of 2C DNA values were compiled from the Plant DNA C-values database maintained at RBG, Kew (prime estimates; Bennett & Leitch, 2005). Species used for analysis comprised 29 orders and 34 families of angiosperms across monocots (32 species), eudicots (62 species), and magnoliids (seven species). 2C DNA estimates ranged 150-fold from 0.539 to 84.9 Gbp. The mean of our monocot sample was larger (2C = 18.35 Gbp) when compared with the means of both magnoliids (2C = 3.23) and eudicots (2C = 5.33 Gbp). There was no appreciable difference in the respective ranges of monocots (2C = 0.686 – 85.0 Gbp) and eudicots (2C = 0.539 – 56.7 Gbp); however, the range in 2C DNA for the magnoliids was much smaller (2C = 1.57 – 9.11 Gbp), although this may reflect the small sample for this group. We also analyzed the data using the monoploid genome size (1Cx DNA amount; Greilhuber *et al.*, 2005), which corresponds to the DNA amount in one chromosome set (x). Thus, the monoploid genome size is calculated by dividing the 2C DNA amount by the level of ploidy (i.e. 2x, 4x, etc).

For each species in our data set, we also classified growth form according to the Glopnet database (Wright *et al.*, 2004) and the Seed Information Database (Flynn *et al.*, 2004). Our data set consisted of 41 herbaceous, 26 shrub, and 34 tree species.

Leaf cell measurements

Measurements were taken from epidermal impressions of the abaxial (lower) and adaxial (upper) surfaces of mature, fully expanded leaves. Epidermal impressions were made using clear nail varnish (Maxiflex^{TM}, Collection 2000, Miami, FL, USA) applied to the middle portion on either side of the midrib and away from the margins. This region of the leaf has been shown to contain guard cell lengths and stomatal densities comparable to the means of the entire leaf (Smith *et al.*, 1989; Willmer & Fricker, 1996; Poole *et al.*, 2000). Epidermal impressions were placed onto a microscope slide and photographed using a QICAM 12-bit Fast 1394 camera (QImaging, Surrey, Canada) mounted to a Leitz Laborlux compound microscope (Leica Microsystems, Wetzlar, Germany). A stage micrometer was used for measurement calibration. Seven measurements of guard cell length, stomatal density, and epidermal cell area were taken in random fields of view. Averages were calculated for three separate individuals per species. Species values were then calculated from the arithmetic mean of the three leaf averages. The abaxial surface measurements were used for all analyses as it is the ancestral character state shared among most angiosperms (Mott *et al.*, 1982; Willmer & Fricker, 1996) and there were only 22 species in our sample with stomata on the adaxial surface of the leaf. However, the relationship between guard cell lengths of the abaxial and adaxial surfaces was nearly 1 : 1 (slope = 0.979; $r^2 = 0.974$; $P < 0.001$).

Guard cell length (μm) and stomatal density (stomata mm^{-2}) measurements were carried out using QCapture Pro 5.0 software (QImaging, Surrey, Canada). We measured guard cell length instead of total guard cell area because of the dynamics of stomatal movement. When stomata open or close the short axis (ventral and dorsal lengths) of the guard cells can increase or decrease but the long axis remains the same (Willmer & Fricker, 1996). Guard cell lengths were measured to the nearest micrometer viewed at ×40 magnification. Stomatal density was estimated by counting the number of stomata per field of view at ×20 magnification. These values were then converted to stomata per mm^2. Epidermal cell area measurements were carried out using ImageJ software (Abramoff *et al.*, 2004).

Phylogenetic tree

We used Phylomatic (tree version: R20050610.new; Webb & Donoghue, 2005) to construct a 'mega-tree' hypothesis for our species sample. This command line program is a compilation of previously published phylogenies and its ordinal 'backbone' and family resolutions are based on the Angiosperm Phylogeny Website (APweb; Stevens, 2006), the best current estimate of relationships of higher plants. The program first matches a species by the genus, then by family. Phylomatic now outputs trees containing within-family resolutions; however, species within a genus are always returned as a polytomy. Phylomatic also outputs 'mega-trees' with branch length information, which are based on the fossil-calibrated molecular diversification estimates of Wikström *et al.* (2001).

Comparative methods

Cross-species analyses We performed a conventional analysis of variance (ANOVA) to test for significant differences in trait means between monocots and eudicots and among varying growth forms. Trait associations without taking into account phylogeny were described by their standardized major axes (SMA; model II regression). SMA analyses are preferred when the purpose is to estimate the best line describing the relationship between two variables (Warton *et al.*, 2006). An SMA is a best-fit line through the centroid of standardized data and rescaled back onto the original axes (Wright *et al.*, 2007). The SMA procedure results in the minimization of the residuals in both the dependent and independent variables. This is particularly useful when it is not known *a priori* which variables should be considered dependent and independent (i.e. does guard cell length predict stomatal density, or vice versa?). For each pair-wise trait combination, we also compared, and tested for a significant difference in slope between, monocots and eudicots, and growth forms. We used a likelihood ratio procedure to test for a common slope for within-group SMA analyses (Warton & Weber, 2002). When a common slope was found we also tested for significant shifts along a common axis (e.g. mean group differences, but same slope estimate). Conventional ANOVA analyses were performed using R (R Development Core Team, 2007). SMA analyses were performed using the (s)matr package (Warton & Ormerod, 2007).

These cross-species results were directly compared with the results obtained from analyses that incorporated phylogenetic information (see below). For trait associations, the use of both analyses can provide a more complete view of the evolutionary relationship between variables. For example, significant cross-species SMA relationships and nonsignificant independent contrasts results can be an indication that deeper divergences (i.e. higher level taxonomic divergences) are greatly influencing the overall relationship (see Felsenstein, 1985; Price, 1997; Ackerly & Reich, 1999; Moles *et al.*, 2005; Beaulieu *et al.*, 2007a,b).

Phylogenetic analyses All analyses that incorporated phylogenetic information were carried out using various programs in the DOS-based computer package pdap (Garland *et al.*, 1993). We used a phylogenetically informed ANOVA to test if significant cross-species trait differences (see previous section) between monocots and eudicots, and between growth forms, were larger than expected based on a random model of Brownian motion evolution (Garland *et al.*, 1993). We used pdsimul to generate 1000 Monte Carlo simulations using our tree topology and the appropriate branch length transformation

(see later in this section). All simulations were carried out under a gradual model of Brownian motion evolution. The simulated data sets were analyzed in PDANOVA to obtain null distributions of the *F*-statistic to compare against the observed *F*-statistic calculated from the cross-species data (see previous section). If the observed *F*-statistic was greater than 95% of the null distribution, then trait differences were greater than expected based on a model of Brownian motion evolution. This analysis was carried out for each trait separately.

Independent contrasts were calculated across our phylogeny using PDTREE (Garland *et al.*, 1999; Garland & Ives, 2000). The method of independent contrasts iteratively calculates trait differences (termed 'contrasts') between extant species pairs, and subsequently their weighted internal node averages, starting at the tips and moving down to the root of a phylogeny (Felsenstein, 1985). These contrasts are then standardized by their branch length information to ensure statistical adequacy (Garland *et al.*, 1992). This calculation transforms the data into $N - 1$ independent data points each representing an evolutionary divergence. To assess whether the use of independent contrasts was appropriate, we used a randomization procedure and calculated a descriptive statistic to test for trait similarity among closely related species (termed 'phylogenetic signal'; Blomberg *et al.*, 2003). Phylogenetic signal is expected under a Brownian motion model of trait evolution and its presence indicates that species values do not satisfy the assumption of independence (Felsenstein, 1985; Blomberg *et al.*, 2003). For the randomization procedure, we calculated the observed variance of the contrast data and compared it to the values obtained from 1000 Monte Carlo simulations that randomized trait data across the tips of the phylogeny (this was carried out in PDRANDOM). If the observed variance was less than 95% of the null distribution, then the observed data exhibited greater phylogenetic signal than random. The descriptive statistic K was used to describe the degree of phylogenetic signal for each trait (Blomberg *et al.*, 2003). Briefly, the K statistic is the ratio of the observed mean square error derived from a phylogenetically corrected mean and the expected mean square error given our tree topology and branch length information assuming Brownian motion. A $K = 1$ would indicate that closely related species have trait values that are similar to those expected given Brownian motion. A $K < 1$ would indicate that closely related species have trait values that are less similar than expected given a Brownian model of evolution. The K statistics were calculated using an R script co-written by David Ackerly and Simon Blomberg (S. Kembel, pers. comm.).

Before testing for trait associations, we first examined the adequacy of our branch lengths in standardizing the contrast data. We calculated the correlation between the absolute value of the standardized contrasts and the corresponding standard deviation (Garland *et al.*, 1992). These diagnostic tests of branch length standardization resulted in different traits requiring different branch length transformations. For 2C DNA content and guard cell length the correlation between the absolute value of the standardized contrasts and the standard deviation was not significant (2C, $P = 0.660$; guard cell length, $P = 0.671$). This indicated that the contrasts were adequately standardized. For stomatal density and epidermal cell area there was a significant and negative correlation (both $P < 0.001$) and a $\log_{10}$ transformation of the branch lengths removed the correlation (epidermal cell area, $P = 0.543$; stomatal density, $P = 0.541$). The proper branch length transformations were used for ANOVA simulations of trait differences (see earlier in this section). However, it was not possible to compare SMA slope estimates obtained from independent contrasts and those that did not incorporate phylogeny. As an analysis of sensitivity to different branch length transformations, we performed SMA analyses between traits with the best branch length transformation for both traits as well as the untransformed branch lengths. All results were significant and SMA slope estimates were very similar regardless of branch length transformation method used ($r^2 = 0.992$). Therefore, we only present SMA results for independent contrasts of all pair-wise trait combinations using the untransformed branch lengths. For each pair-wise trait combination, we also compared, and tested for significant differences in slope estimates between, monocots and eudicots, and growth forms. Soft polytomies (i.e. phylogenetic uncertainty) were randomly resolved and given a zero branch length. This resulted in 21 branches with a length of zero. Significance testing was initially carried out using the bounded degrees of freedom approach of Purvis & Garland (1993; also see Garland & Diaz-Uriarte, 1999). All results were either significant or not significant regardless of degrees of freedom used. Thus, significance is reported as calculated using the maximum degrees of freedom. We utilized the output of our standardized contrasts from PDTREE and the R package (S)MATR (Warton & Ormerod, 2007) to obtain slope estimates and r^2 from SMA analyses forced through the origin (Garland *et al.*, 1992). We also carried out the same likelihood ratio procedure as described earlier in this section to test for a common slope for within-group SMA analyses (Warton & Weber, 2002).

Results

Cross-species

There was a considerable range of guard cell and epidermal sizes among species (Fig. 1) and analysis across all species showed that 2C DNA content was significantly and positively associated with guard cell length and epidermal cell area ($r^2 = 62$ and 59%, respectively; Table 1, Fig. 2a,b). There was a significant negative association between 2C DNA content and stomatal density, but 2C DNA explained less of the variation in stomtatal density ($r^2 = 34\%$; Table 1, Fig. 2c) compared with guard cell length and epidermal cell area.

Analyses comparing monocots and eudicots showed that 2C DNA content and leaf cell traits varied along a common slope for both groups, with eudicots clustering together with

Table 1 Standardized major axis (SMA) slope estimates describing the relationships among 2C DNA, epidermal cell area, guard cell length, and stomatal density for both cross-species (regression without incorporating phylogeny) and independent contrasts analyses

	Epidermal cell area (μm^2, log)						Guard cell length (μm, log)						Stomatal density (stomata mm^{-2}, log)					
	Regression			Independent contrasts			Regression			Independent contrasts			Regression			Independent contrasts		
	r^2	Slope	95% CI	r^2	Slope	95% CI	r^2	Slope	95% CI	r^2	Slope	95% CI	r^2	Slope	95% CI	r^2	Slope	95% CI
2C DNA content (Gbp, log)																		
All species	0.59	0.74	(0.65, 0.84)	0.19	0.92	(0.77, 1.09)	0.620	0.248	(0.22, 0.28)	0.41	0.29	(0.25, 0.34)	0.339	–0.614	(–0.52, –0.72)	0.144	–0.817	(–0.68, –0.98)
Monocots	0.56	0.66	(0.52, 0.84)	0.27	0.79	(0.58, 1.08)	0.640	0.234	(0.19, 0.29)	0.56	0.25	(0.20, 0.32)	0.312	–0.559	(–0.41, –0.76)	0.190	–0.723	(–0.52, –1.01)
Eudicots	0.48	0.76	0.63, 0.92)	0.13	1.05	(0.83, 1.33)	0.558	0.267	(0.23, 0.32)	0.30	0.33	(0.27, 0.41)	0.251	–0.534	(–0.43, –0.67)	0.108	–0.889	(–0.70, –1.13)
Trees	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Shrubs	0.33	0.75	(0.53, 1.05)	NS	NS	NS	0.462	0.268	(0.20, 0.36)	0.32	0.32	(0.23, 0.45)	NS	NS	NS	NS	NS	NS
Herbs	0.53	0.61	(0.49, 0.76)	0.27	0.76	(0.57, 0.99)	0.664	0.244	(0.20, 0.29)	0.56	0.26	(0.21, 0.32)	0.200	–0.591	(–0.44, –0.79)	0.158	–0.710	(–0.53, –0.95)
Epidermal cell area (μm^2, log)																		
All species	–	–	–	–	–	–	0.631	0.337	(0.30, 0.38)	0.32	0.32	(0.27, 0.38)	0.612	–0.835	(–0.74, –0.95)	0.436	–0.893	(–0.77, –1.04)
Monocots	–	–	–	–	–	–	0.535	0.355	(0.28, 0.46)	0.35	0.32	(0.24, 0.43)	0.604	0.847	(–0.67, –1.07)	0.550	–0.918	(–0.72, –1.18)
Eudicots	–	–	–	–	–	–	0.631	0.351	(0.30, 0.41)	0.30	0.32	(0.26, 0.39)	0.525	–0.702	(–0.59, –0.84)	0.368	–0.846	(–0.69, –1.04)
Trees	–	–	–	–	–	–	0.371	0.370	(0.28, 0.47)	0.32	0.29	(0.22, 0.39)	0.476	–0.909	(–0.70, –1.18)	0.571	–0.951	(–0.75, –1.20
Shrubs	–	–	–	–	–	–	0.580	0.359	(0.27, 0.47)	0.15	0.33	(0.23, 0.48)	0.358	–0.753	(–0.54, –1.05)	0.215	–0.796	(–0.55, –1.15)
Herbs	–	–	–	–	–	–	0.545	0.400	(0.32, 0.50)	0.36	0.34	(0.26, 0.44)	0.475	–0.970	(–0.77, –1.22)	0.511	–0.940	(–0.75, –1.18)
Guard cell length (μm, log)																		
All species	–	–	–	–	–	–	–	–	–	–	–	–	0.570	–2.48	(–2.18, –2.82)	0.386	–2.79	(–2.39, –3.26)
Monocots	–	–	–	–	–	–	–	–	–	–	–	–	0.633	–2.38	(–1.91, –2.98)	0.446	–2.88	(–2.19, –3.79)
Eudicots	–	–	–	–	–	–	–	–	–	–	–	–	0.499	–2.00	(–1.67, –2.40)	0.357	–2.67	(–2.17, –3.28)
Trees	–	–	–	–	–	–	–	–	–	–	–	–	0.372	–2.46	(–1.86, –3.26)	0.256	–3.30	(–2.43, –4.48)
Shrubs	–	–	–	–	–	–	–	–	–	–	–	–	0.430	–2.10	(–1.54, –2.87)	0.529	–2.43	(–1.83, –3.23)
Herbs	–	–	–	–	–	–	–	–	–	–	–	–	0.474	–2.43	(–1.92, –3.06)	0.417	–2.77	(–2.17, –3.54)

The r^2 and the slope are shown for SMA using independent contrasts analyses. All SMA slopes of independent contrasts were forced through the origin.
NS, nonsignificant; all other results $P < 0.001$.
CI, confidence interval.

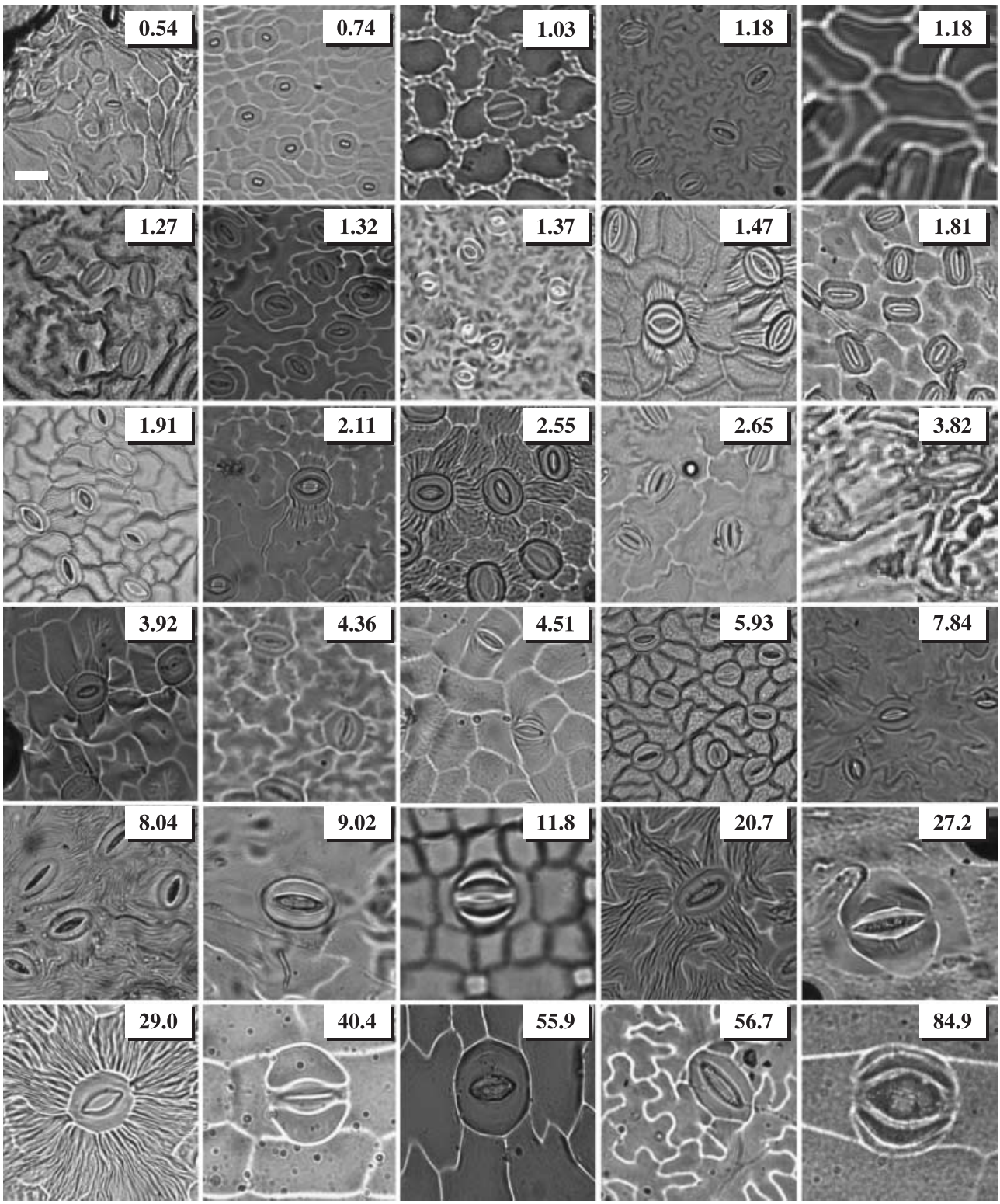

Fig. 1 Abaxial leaf epidermis images showing guard cell size in relation to 2C DNA amount. Numbers in each box correspond to the 2C DNA amount. All images were taken at ×40 magnification. Bar, 20 μm.

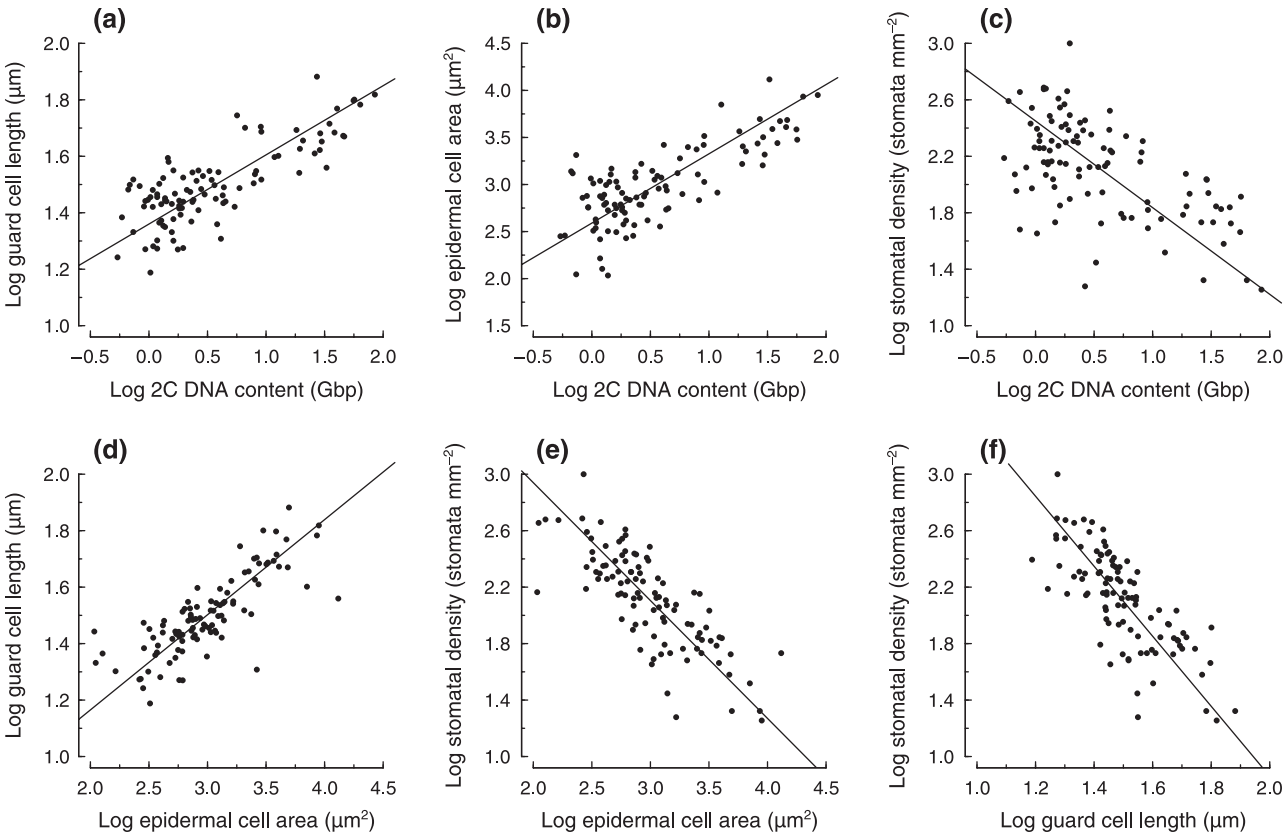

Fig. 2 Scatter plot of the significant cross-species relationships (without considering phylogeny) between all pair-wise trait combinations (see Table 1). All slope estimates are the standardized major axis (SMA; model II regression) describing the best-fit line from minimizing residuals in both dependent and independent variables.

lower trait values. When analyses were partitioned across growth forms, herbaceous species showed comparable slope estimates to those calculated across all species (Table 1). However, interestingly, relationships within shrubs and trees were not significant for 2C DNA content and guard cell length, epidermal cell area, and stomatal density. In all the above cases, results for 1Cx were very similar (but slightly weaker) when compared with results for 2C DNA content.

Relationships among leaf traits were all highly significant (Table 1, Fig. 2d–f). For example, both epidermal cell area and guard cell length were negatively associated with stomatal density (Table 1, Fig. 2 e,f). The slope estimates for all pair-wise leaf cell trait comparisons were similar and not significantly different between monocots and eudicots, or between growth forms (Table 1).

Phylogenetic signal and independent contrasts

Closely related species were more similar than would be expected by chance, indicating there is phylogenetic signal for all traits. 2C DNA content exhibited a stronger degree of phylogenetic signal ($K = 0.959$) than did guard cell length ($K = 0.685$), epidermal cell area ($K = 0.630$), or stomatal density ($K = 0.540$). Therefore, because of the phylogenetic signal in our data set, we used independent contrasts for further analyses.

The slope estimates obtained from independent contrasts analyses were significantly greater in magnitude but had a lower r^2 when compared with cross-species results (slope = 1.10; 95% confidence interval (CI) 1.05–1.15). Partitioning the analyses for eudicots, for monocots, or within each growth form did not lead to differences in r^2 or magnitude (Table 1). However, independent contrasts for trees and shrubs still did not uncover any significant relationships between 2C DNA content and leaf cell traits, but there were significant relationships between leaf cell traits (excluding 2C DNA content; Table 1). All pair-wise trait relationships within herbaceous species were significant.

Trait differences

Monocots had a greater mean genome size (both 2C and 1Cx DNA), guard cell length, and epidermal cell area when compared with eudicots. Mean stomatal density was also significantly lower in monocots compared with eudicots. However, phylogenetically corrected ANOVA suggested that the mean values for monocots and eudicots were significantly

	Conventional ANOVA		Monte Carlo simulation	
Source	Observed *F*	*P*	Critical value	*P*
Log_{10} 2C DNA				
Clade	18.9	< 0.001	79.8	0.351
Growth form	48.9	< 0.001	18.6	< 0.001
Log_{10} guard cell length				
Clade	10.5	0.001	76.3	0.458
Growth form	31.1	< 0.001	19.3	0.009
Log_{10} epidermal cell area				
Clade	24.8	< 0.001	137.8	0.282
Growth form	68.5	< 0.001	24.8	< 0.001
Log_{10} stomatal density				
Clade	31.2	< 0.001	149.7	0.223
Growth form	43.7	< 0.001	26.5	0.002

Table 2 Results from a conventional analysis of variance (ANOVA) and Monte Carlo simulation to test for significant trait differences between monocots and eudicots (clade), and among trees, shrubs, and herbs (growth form), relative to those expected based on random Brownian motion evolution

The critical value is the 95th percentile obtained from a distribution of 1000 Monte Carlo simulated *F*-statistics assuming a gradual model of Brownian motion evolution.
P-values from Monte Carlo simulations are the proportion of simulated *F*-statistics that are greater than the observed *F*-statistic using conventional ANOVA.
Monte Carlo simulations were carried out using log_{10}-transformed branch lengths for epidermal cell area and stomatal density (see text).

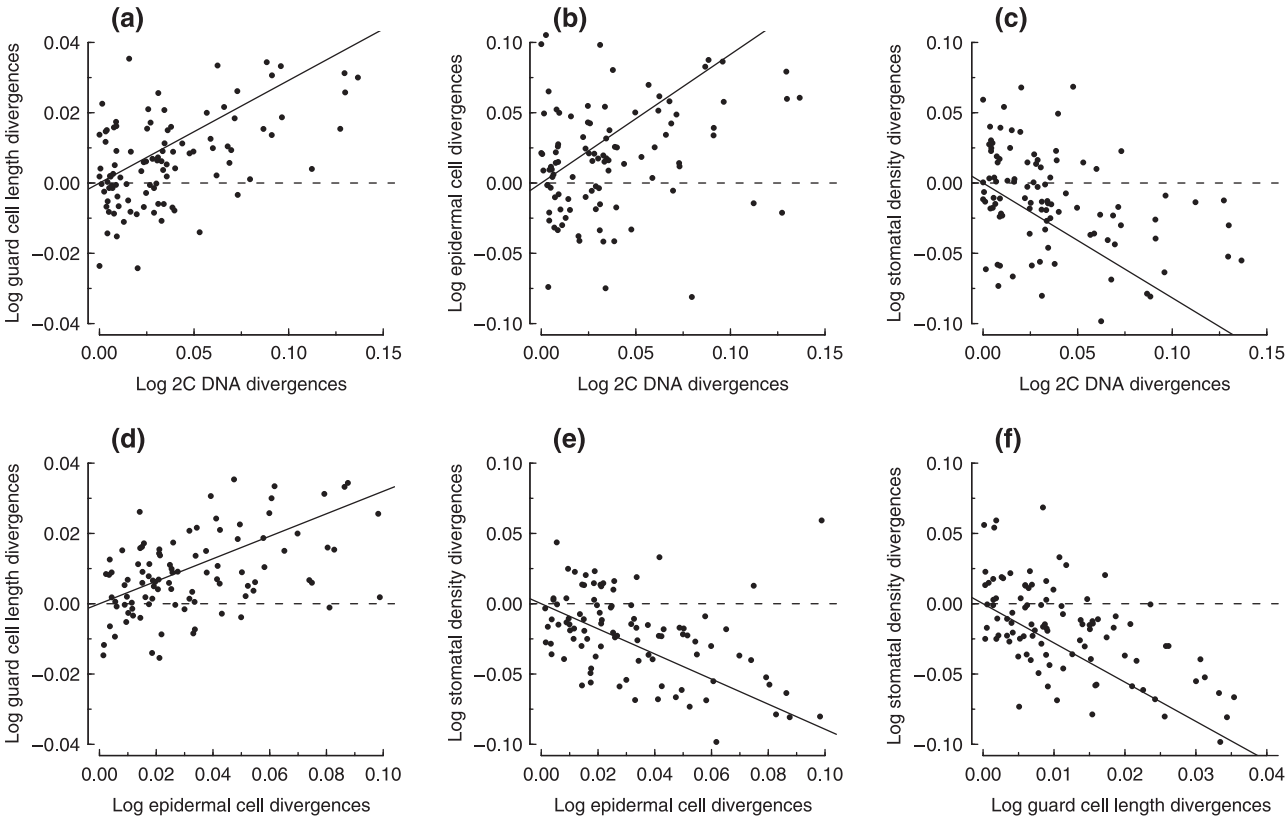

Fig. 3 Contrast plots depicting the significant relationships for all pair-wise trait combinations (see Table 1). All slope estimates are the standardized major axis (SMA; model II regression) describing the best-fit line from minimizing residuals in both dependent and independent variables. All SMA lines were forced through the origin (Garland *et al*., 1992).

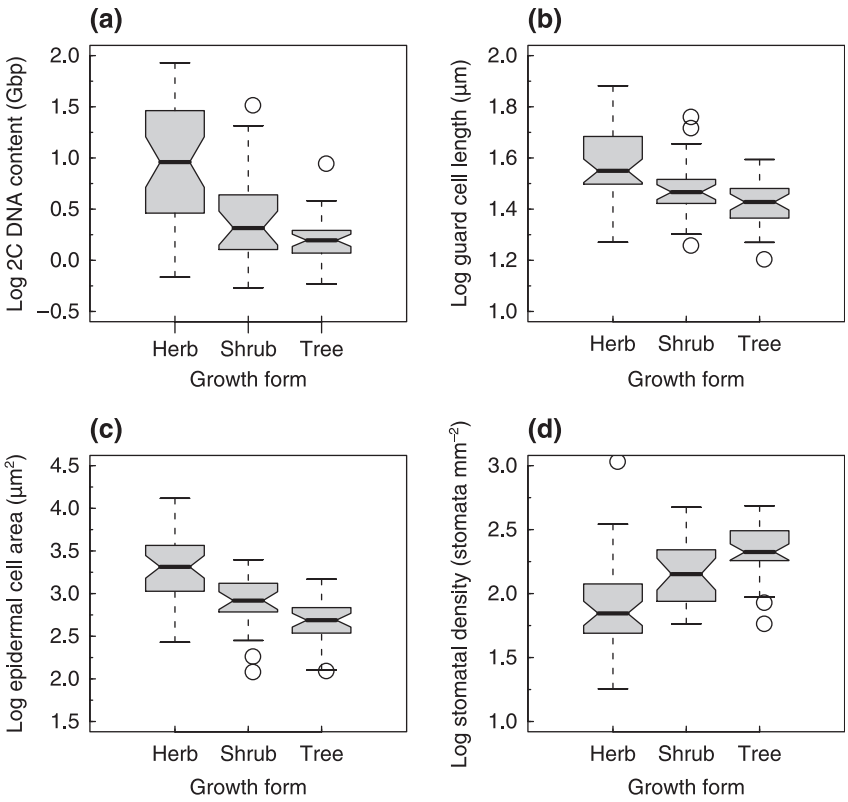

Fig. 4 The relationship between growth form and (a) 2C DNA content, (b) guard cell length, (c) epidermal cell area, and (d) stomatal density. The box plots represent the median (central line), first and third quartiles (gray box), and outliers. Median line notches that do not overlap indicate significant differences between growth forms. There are significant evolutionary differences among growth forms for all four traits (see Table 2), where trees and shrubs have smaller 2C DNA values, smaller cells (i.e. guard and epidermal cells) and higher stomatal densities than do herbaceous growth forms.

different but not more different than would be expected given a model of Brownian motion evolution (Table 2). In other words, the difference observed between the two clades could have arisen by chance. Among growth forms, trees and shrubs had significantly smaller genome sizes, smaller cells (guard and epidermal cells), and higher stomatal density than herbaceous species (Fig. 4). In addition, after incorporating both chance and phylogeny in the ANOVA, we found that all trait values among growth forms varied significantly more than expected given a random model of Brownian motion (Table 2, Fig. 4).

Discussion

The main purpose of this study was to re-examine the relationship between genome size and cell size within angiosperms using a large species set and a comparative approach. Across 101 species of varying growth forms, one of the most striking results is the remarkable linearity (on a log-transformed scale) in the relationship between genome size and cell size. There is a steady progression of species with larger genomes with increasingly larger cells (Figs 1, 2a,b). Moreover, we found that across all species genome size explains nearly 60% of the total variation in both guard cell length and epidermal cell area. Tests of phylogenetic signal indicated that this pattern was not independent of ancestry; however, even after incorporating phylogenetic history, slope estimates were similar to those found using conventional statistics (Table 1). Thus, we found not only a strong association across extant species (regression results) but also strong correlated evolution (independent contrasts results) between genome size and cell size. The strength of the relationship was growth form dependent. Despite nonsignificant associations between genome size and cell size within trees, trees were characterized by having small genome sizes and cell sizes with decreased variance within the group compared with other growth forms (Fig. 4). Therefore, our results provide support for the general assumption that genome size evolution (whether towards smaller or larger size) is a strong predictor of the *minimum* size of any given cell type (Bennett, 1972; Gregory, 2001). Additional factors such as the influence of individual genes (e.g. *Too Many Mouths* (*TMM*); Nadeau & Sack, 2002) and environmental conditions must also play an important role in determining cell size, but perhaps only by modulating the final cell size from the minimum set by DNA content. A specific model clarifying the mechanism for this relationship is needed.

Among stomatal traits, there was also a general congruency between cross-species and independent contrasts results for all pair-wise comparisons. Moreover, these slope estimates for leaf cell traits were also congruent within each of the three growth forms, despite significant evolutionary differences in stomatal traits among trees, shrubs, and herbs (Table 1, Fig 4). These results may signal general functional constraints coordinating the evolution of stomatal traits (Hetherington & Woodward, 2003; Kerkhoff *et al.*, 2006). The number and

subsequent expansion of epidermal cells influence stomatal density through compensatory mechanisms associated with cell size and cell number (Salisbury, 1927; Beerling & Chaloner, 1993; Weijschedé *et al.*, 2008). The coordination of the size and frequency of stomata is thought to signify an optimal balance of carbon fixation per unit of water lost across many different environments. Large and significant changes to genome size could alter the water use efficiency. For example, within herbaceous species the evolution of larger genome sizes and larger cell sizes (guard cell length and epidermal cell area) was associated with a decrease in stomatal density. If genome size sets the minimum size of both guard cells and epidermal cells, the resulting change in stomatal density may predispose a species to a particular ecological and life-history strategy. In dry environments, smaller stomata allow a rapid response to water stress, while high densities allow maximization of CO_2 diffusion during optimal photosynthetic conditions (Aasamaa *et al*, 2001; Hetherington & Woodward, 2003). Large genomes are never associated with this trait combination and therefore may be limited in their response to water stress and high temperature. Knight & Ackerly (2002) have shown that large-genome species are less frequent in environments characterized by low precipitation and high temperatures.

Trees tend to have small genome size and small, dense stomata. Interestingly, within the tree sample, there is no significant relationship between genome size and any of these cell traits (Table 1). However, when these data are superimposed on the entire data set, significant relationships emerge (Figs 2a–c, 3a–c). The small cells and generally high stomatal density found in trees may have adaptive significance. Increased stomatal density is associated with greater stomatal conductances and transpiration rates, which are thought to be necessary for moving water and nutrients through longer xylem pathways (Woodward, 1998). In addition, smaller stomata allow greater stomatal resistance and stomatal control during water stress conditions (Aasamaa *et al.*, 2001; Hetherington & Woodward, 2003). Thus, we expect that large and significant increases in DNA content might negatively impact trees by decreasing stomatal control of water loss, which may represent another ecological constraint on large-genome species (Knight *et al.*, 2005). Consistent with this hypothesis, polyploidy is rare among angiosperm trees (Stebbins, 1938; Ancel Meyers & Levin, 2006). Conversely, genome size evolution may also be generally slower in angiosperm trees because of longer generation times.

Leaf cell traits, including cell size, exhibited less phylogenetic signal than did genome size. That is, closely related species were less similar in their stomatal trait values than expected under a random model of Brownian motion evolution. Deviations from the expected phylogenetic signal (i.e. $K = 1$) can be a result of an adaptive response to selection and/or the inclusion of several sources of error, such as tree topology, branch length information, or species measurements (Blomberg & Garland, 2002; Blomberg *et al.*, 2003; Ives *et al.*, 2007). There are certainly potential errors in our phylogeny given that it is mostly resolved to family level and aged using interpolated branch lengths from a small sample of divergence time estimates (Wikström *et al.*, 2001). However, errors attributed to phylogeny should generally reduce phylogenetic signal among all traits (Rezende *et al.*, 2004). Yet, consistent with studies reported for various clades of angiosperms (Albach & Greilhuber, 2005; Weiss-Schneeweiss *et al.*, 2005; Leitch *et al.*, 2007), our genome size sample showed phylogenetic signal very near the expectation assuming random Brownian motion ($K = 0.959$).

While we do not discount the presence of various forms of error, selection may also contribute to the reduction in phylogenetic signal exhibited by stomatal traits (Blomberg & Garland, 2002; Blomberg *et al.*, 2003). There is a recognized functional link between stomatal density and atmospheric CO_2 (McElwain & Chaloner, 1995; Beerling & Woodward, 1997; Beerling *et al.*, 2001). The steady decline in atmospheric CO_2 over the last 200 Myr (Crowley & Berner, 2001) has been associated with an overall increase in stomatal density, which from our results implies declining guard cell length and epidermal cell area (Table 1, Figs 2, 3). Moreover, the stomatal response to environmental change can also be rapid, occurring on 100-yr timescales (Royer, 2001). Thus, the large discrepancy in the degree of phylogenetic signal between genome size and stomatal traits may have biological significance. Perhaps environmental factors that influence stomata do not directly influence genome size variation. Instead, genome size may generally evolve stochastically (i.e. Oliver *et al.*, 2007; Leitch *et al.*, 2007) but can impose a limit to the response of stomata to environmental factors (Knight & Ackerly, 2002; Knight *et al.*, 2005). While this is intriguing, more work is needed to examine whether it is true for all cell types.

Taken together, results from animals and plants suggest that the relationship between genome size and cell size is a universal phenomenon. The robustness of the relationship will make it possible to infer genome size from fossil plant specimens, just as Organ *et al.* (2007) used osteocyte cell size in fossil dinosaurs to infer that the small genome size of birds was a pre-existing trait within the saurischian dinosaur lineage. Leaf impression fossils with well-defined guard cells are common in the fossil record for plants, and therefore, based on the results presented here, we suggest ancestral genome sizes could be inferred for early land plants (Leitch, 2007). Extending this work further could examine how genome size responds to climatic catastrophes (e.g. the KT extinction event). This type of analysis will provide further insight into the tempo of genome size evolution.

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