Links between environment and stomatal size through evolutionary time in Proteaceae

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Abstract

The size of plant stomata (adjustable pores that determine uptake of CO_2 and loss of water from leaves) is considered to be evolutionarily important. This study uses fossils from the major southern hemisphere family Proteaceae to test whether stomatal cell size responded to Cenozoic climate change. We measured the length and abundance of guard cells (the cells forming stomata), the area of epidermal pavement cells, stomatal index and maximum stomatal conductance from a comprehensive sample of fossil cuticles of Proteaceae, and extracted published estimates of past temperature and atmospheric CO_2. We developed a novel test based on stochastic modelling of trait evolution to test correlations among traits. Guard cell length increased, and stomatal density decreased significantly with decreasing palaeotemperature. However, contrary to expectations, stomata tended to be smaller and more densely packed at higher atmospheric CO_2. Thus, associations between stomatal traits and paleoclimate over the last 70 MY in Proteaceae suggest that stomatal size is significantly affected by environmental factors other than atmospheric CO_2. Guard cell length, pavement cell area, stomatal density, stomatal index covaried in ways consistent with coordinated development of leaf tissues.

1. Introduction

The epidermis of plant leaves contains stomata, tiny adjustable pores that regulate diffusive conductance, and therefore the loss of water and uptake of CO_2 for photosynthesis. These functionally significant structures can be observed directly on many leaf fossils, and provide potential proxies for several aspects of past environments [1]. Thus, the size of fossil guard cells (the cells that form stomata) has been used to estimate past genome size [1]. The number of stomata per unit area (stomatal density), stomatal index (which reflects the ratio of stomata to epidermal pavement cells), and models employing stomatal density and stomatal size have been used to estimate past levels of atmospheric CO_2 [2-4]. Recently, attention has focused on the importance of stomatal size in plant evolution [5-9].

Along with stomatal density, stomatal size affects maximum stomatal conductance – a major determinant of maximum photosynthetic assimilation [10]. Although a large stoma has greater conductance than a small one [11], species with large stomata tend to have fewer stomata resulting in lower total conductance than those with small stomata, both overall and through the focal group of this study, Proteaceae [5, 7, 12, 13]. Contemporary models of stomatal response to atmospheric CO_2 show that higher CO_2 should correspond to lower stomatal conductance [14], and thus favour larger stomata [12]. Such a relationship is supported by fossil evidence showing positive correlation between stomatal size and atmospheric CO_2 over the last 395 million years [9, 12].

However, stomatal size may be under strong environmental selection independent of atmospheric CO_2. In Proteaceae, an important Southern Hemisphere woody plant family, large guard cells (and therefore stomata) are associated with open vegetation and small guard cells and greater stomatal density are linked with rainforest (closed forest) [15].

Fossils from the Cenozoic (the last 66 million years) provide opportunities to examine the drivers of stomatal size through evolutionary time. Major, and often global, changes in environment through this period are likely to have altered the selective regimes operating on stomata. For example,

compared to current day conditions, the Early Eocene ~56 – 47.6 Ma (million years ago), was characterised by considerably higher temperatures [16] and atmospheric CO_2 [17, 18], much wetter climates and greater amounts of forest cover in the study region of this current work, Australasia [19]. Fossil Proteaceae leaves provide an exceptional resource for examining the impacts of atmospheric change on stomatal characteristics because sediments in southern Australia preserve an extraordinary richness of Proteaceae fossils that span critical atmospheric changes in the Cenozoic period. A long history of study of Proteaceae cuticles [20] means that they can be confidently identified as members of this family, thus providing a sufficient sample size to allow clear insight into the impact of evolutionary change over the last 65 million years in this important lineage.

In this paper, we study whether temporal trends in guard cell length and other relevant epidermal cell characteristics in Proteaceae are predicted by environmental change. We use fossils representing 116 site by species combinations to test whether changes in stomatal traits are correlated with major trends in environment through the Cenozoic. To do this we tested the correlations of fossil stomata and epidermal pavement cells with estimates of past atmospheric CO_2 and temperature by comparing them with null models based on simulated "fossils" derived from simulated trees. That analysis was necessary because tests of correlations between characters are affected by phylogenetic relationships [21].

2. Methods

We analysed all known late Cretaceous to mid Miocene (~70 - 13 Ma) Proteaceae fossil leaf specimens with preservation of stomatal morphology [22], representing 116 species by site combinations (electronic supplementary material, tables S1and S2). The specimens came from Australia and New Zealand. The fossils are likely to come from canopy leaves, where stomatal resistance is likely to outweigh boundary layer resistance: Proteaceae do not include specialists of closed forest understoreys, and in general, understorey leaves are poorly represented in the fossil record [23]. All fossils were placed within the family based on phylogenetically informative

characters [20]. Because of homoplasy and the limited number of characters available only some fossils could be assigned to groups within the family. No relevant fossils of late Miocene or Pliocene (~13 to 2.59 Ma) age were available and we excluded younger fossils because of the extreme variability of climate and atmospheric CO_2 during that period. The age ranges of the fossil sites were based on published sources or determined specifically for this project (electronic supplementary material, tables S1 and S3).

We also measured epidermal characters from 109 extant species (electronic supplementary material, table S4) representing 69 of the 80 genera of Proteaceae, with multiple species of large genera. Replicate specimens (measured for most species) indicated that within-species variation in epidermal characteristics was small relative to among-species variation.

Five epidermal characters (electronic supplementary material, table S5 and figure S1) were estimated on both fossils and extant specimens. Guard cell length, a simple, reliable measure of stomatal size on fossils, was measured from all specimens. Stomatal density, stomatal index [100 x number of stomata/(number of epidermal pavement cells + number of stomata)], pavement cell area and theoretical maximum stomatal conductance ($g_{s\ max}$) were calculated for most species by site combinations, based on an average of up to 5 specimens. Measurements were made from digital micrographs of cuticles, using ImageJ version 1.48 (National Institutes of Health, Bethesda, Maryland; http://rsbweb.nih.gov/ij/). $g_{s\ max}$ was estimated following the equation of Parlange and Waggoner [11], assuming that stomatal pore length was 2/3 of guard cell length, stomatal pore width was 1/3 of pore length and stomatal pore depth was the same as stomatal pore length. These assumptions are consistent with dimensions observed in extant Proteaceae (G. J. Jordan unpublished data).

(a) Data analysis

Our 109 extant species could be associated with 71 of the tips in the genus-level dated phylogeny employed here [24]. Because our data included multiple species for many tips, for each tip

representing multiple species we generated a simulated tree for those species using *sim.bd.taxa* in the library *TreeSim* version 2.4 [25, 26] in R [27] with a speciation rate of 1 and an extinction rate of 0.1, scaled that tree to the length of the branch leading to the tip of interest, and then replaced the branch leading to the tip of interest with the simulated tree. We repeated this 1000 times, and present median values for each trait.

Temporal trends in characteristics measured on fossils were identified using generalised additive models with thin-plate smoothing splines, assuming a Gaussian distribution, three knots, and implemented using the *gam* function of the library *mgcv* [28] in R. For analysis, guard cell length, pavement cell area, stomatal density and maximum stomatal conductance were log transformed. Each observation represented a species by site combination, with age attributed to the mid-point of the age-range. Although the fossils may represent a biased sample of the species present in the source vegetation and across the broader landscape, we assume that these biases are relatively constant through time and therefore should not affect our inferences significantly.

(b) Associations between epidermal characteristics and environment

To identify associations between epidermal characteristics and environment we estimated Pearson correlations of fossil epidermal characters with estimators of past global environmental traits – trends in deep ocean temperature [29] and two sets of estimates of levels of atmospheric CO_2 [17, 18]. One of the CO_2 models (Anagnostou) [18] was derived independently of stomatal traits but only spans the Eocene, whereas the other (Beerling and Royer) [17] spans the full Cenozoic, but includes information from fossil stomata. The Anagnostou data were smoothed and interpolated using generalised additive model (as described above), whereas the other data were already smoothed.

Standard tests of correlations between fossil traits and environment (i.e. those that ignore temporal differences in evolved traits) may not be valid for this problem because phylogenetic relationships affect tests of correlations between characters [21]. Thus, Felsenstein [21] highlighted that the non-

independence of traits linked phylogenetically can result in inflated estimates of significance. We therefore used Monte-Carlo simulations to test whether observed correlations were stronger than could be expected by chance. Thus, we compared the observed correlations with correlations between the environmental parameters and trait values of "fossils" simulated on trees comparable to that of Proteaceae. To create the simulated fossils and its corresponding environmental value we followed these steps:

(1) We simulated phylogenetic trees of similar size (1700 tips) as Proteaceae and scaled them to have a crown age of similar to that of Proteaceae - 93.2 million years [24]. On each tree we simulated a trait under an Ornstein-Uhlenbeck model, which is considered to better describe character evolution than Brownian motion [30]. The trees were simulated using birth death processes under a range of values of μ/λ (relative extinction rate, or 1/the birth:death ratio). Extinct branches were retained. We were particularly interested in μ/λ because there is abundant fossil evidence indicating high levels of extinction in Proteaceae [31]. Extinction may have large impacts on tree shape (trees will have relatively longer deep branches as the extinction rate increases relative to the speciation rate [32]). In turn this can affect tests of correlations. If the extinction rate is large compared to the speciation rate then internal nodes will tend to occur near the tips of the tree [33]. The effect of this is to reduce the average distance between tips of the tree (or points on the tree at any time point) and therefore to increase the covariance of traits at these tips. We used nine values of μ/λ (relative extinction rate, or 1/the birth:death ratio) ranging from 0.01 to 0.9 and the Ornstein-Uhlenbeck parameter α set to a value estimated for extant tip data for the relevant parameter (0.0698 for log of guard cell length, 0.3297 for stomatal index, 0.1035 for log of stomatal density, 0.0733 for log of pavement cell area and 0.1687 for $g_{s\,max}$). The trait lability parameter θ was held constant at 1 because θ is independent of α and should not affect the correlations. We simulated 10,000 trees for each combination of μ/λ and α. The trees and traits were simulated using the *fasttree* R function developed for this study. The traits simulated with *fasttree* were comparable to traits simulated using a standard post hoc approach implemented in the *rTrait* function of

TreeSim [26]: for each combination of μ/λ and α employed above (1000 replicates) the mean values of OU parameters (as estimated by *phylolm* [34]) for our traits were within 0.5% of those simulated by *rTrait* (electronic supplementary material, table S6).

(2) For each true fossil, we created a comparable fossil trait value from each simulated tree. To do this, we identified the value of the simulated trait on each edge of the tree present at the age of the true fossil, and randomly selected one of these values. To allow for uncertainty in age estimates for these analyses, the age of each fossil site was permitted to randomly vary within a uniform distribution from the minimum to maximum age. The value of the environmental parameter for that fossil was the value of that parameter (see above) at that time. Thus, if we had Y fossils from time X, we selected Y lineages (including extinct lineages) at time X from the tree, with environmental parameter scores and trait value for that time.

Following standard approaches in stochastic modelling [35], *p*-values under the null hypothesis were calculated as the proportion of simulated correlations more extreme than the observed correlation.

We further investigated the relationship between fossil epidermal traits and past environment with multiple regression using the stats function *lm* in R. For each stomatal trait we fitted each combination of the three environmental predictors, and used AICc (calculated using the package *MuMin* [36] in R) to determine the relative goodness of fit of the models. To facilitate comparisons, we restricted the data to the Eocene, for which complete data was available for all variables.

3. Results

The range in stomatal morphologies of fossil Proteaceae fell within the range observed among living species of that family. Guard cell length (our proxy for stomatal size) of the fossils varied significantly through time, with a small decrease from ~71 Ma (late Cretaceous) to ~55 Ma (earliest Eocene), followed by a strong increase until ~30 Ma (Oligocene), then a slight decrease until ~ 15

(mid-Miocene) (figure. 1). The increase between 55 and 30 Ma had high confidence because of strong representation of fossils. The pattern for stomatal density followed an almost mirror image of this pattern, except for a stronger excursion during the Miocene (figure 1). The temporal trend for other traits were not significant, although pavement cell area showed similar trends to guard cell length, and stomatal index and $g_{s\ max}$ showed similar trends to stomatal density (figure 1).

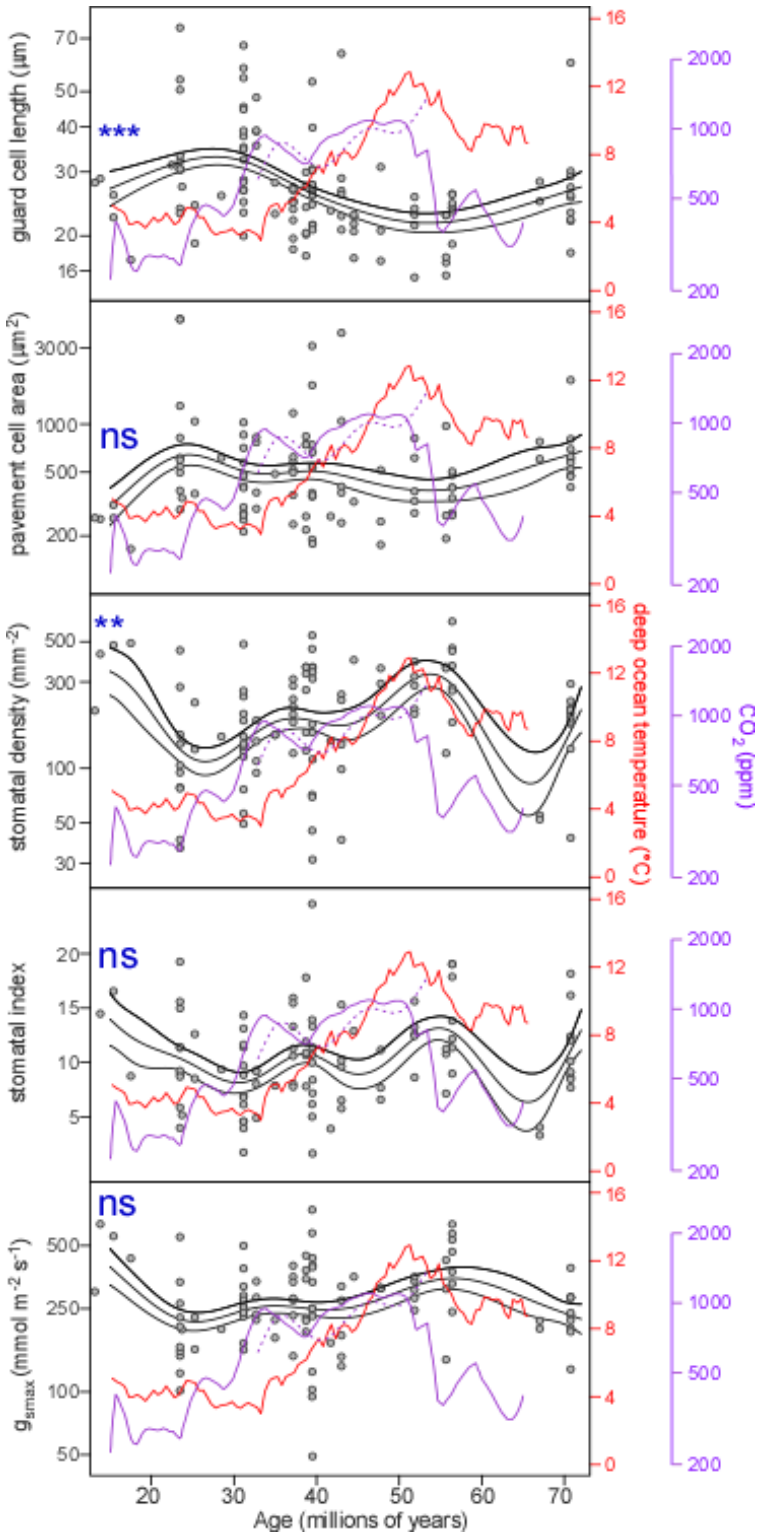

Figure 1. Epidermal characteristics observed on fossils. Trends through time are shown as thin-plate smoothing splines (thick line) ± 1 standard error (fine lines), as estimated using generalised additive models. Significance of variation in stomatal traits through time is shown: *** = $p < 0.0001$; ** = $p < 0.01$; ns = $p > 0.05$. Estimated deep ocean temperatures (red) [29] and levels of atmospheric CO_2 (solid purple for estimates of Beerling and Royer [17], dashed purple line for those of Anagnostou [18]) are overlain.

The estimated significance of the correlations between epidermal traits on fossils and environment decreased as the ratio of extinction to speciation (μ/λ) increased (electronic supplementary material, figure S2), and was always less significant than standard tests not accounting for phylogeny (electronic supplementary material table S7). Under the null hypothesis of no relationship between a trait and an environmental variable, simulations with high relative ratios of extinction to speciation produced a broader range of correlations. We hypothesise that shorter average distances between points on the tree for simulations with high ratios of extinction to speciation [33] increases the covariance between the tips, and therefore reduces the effective sample size. This makes it more likely to see large correlations by chance alone. However, tests were consistent qualitatively, with non-significant tests ($p > 0.05$) remaining non-significant and significant tests ($p < 0.05$) remaining significant regardless of μ/λ, except that the relationship of log of stomatal density with temperature became non-significant at exceptionally high μ/λ (>0.96). Estimated deep ocean temperature [29] showed a highly significant negative correlation ($p < 0.01$) with log of guard cell length and a significant positive correlation with log stomatal density (figure 2), the latter presumably because of the well established negative relationship between these epidermal characteristics [5, 7, 12, 13]. Log of guard cell length showed a significant ($p < 0.05$) correlation with atmospheric CO_2 as estimated by Anagnostou et al., presumably because of the correlation between this measure of atmospheric CO_2 and temperature [18]. Other correlations were not significant, but those of atmospheric CO_2 with log of guard cell length and log of $g_{s\,max}$ were negative or close to zero (figure 2).

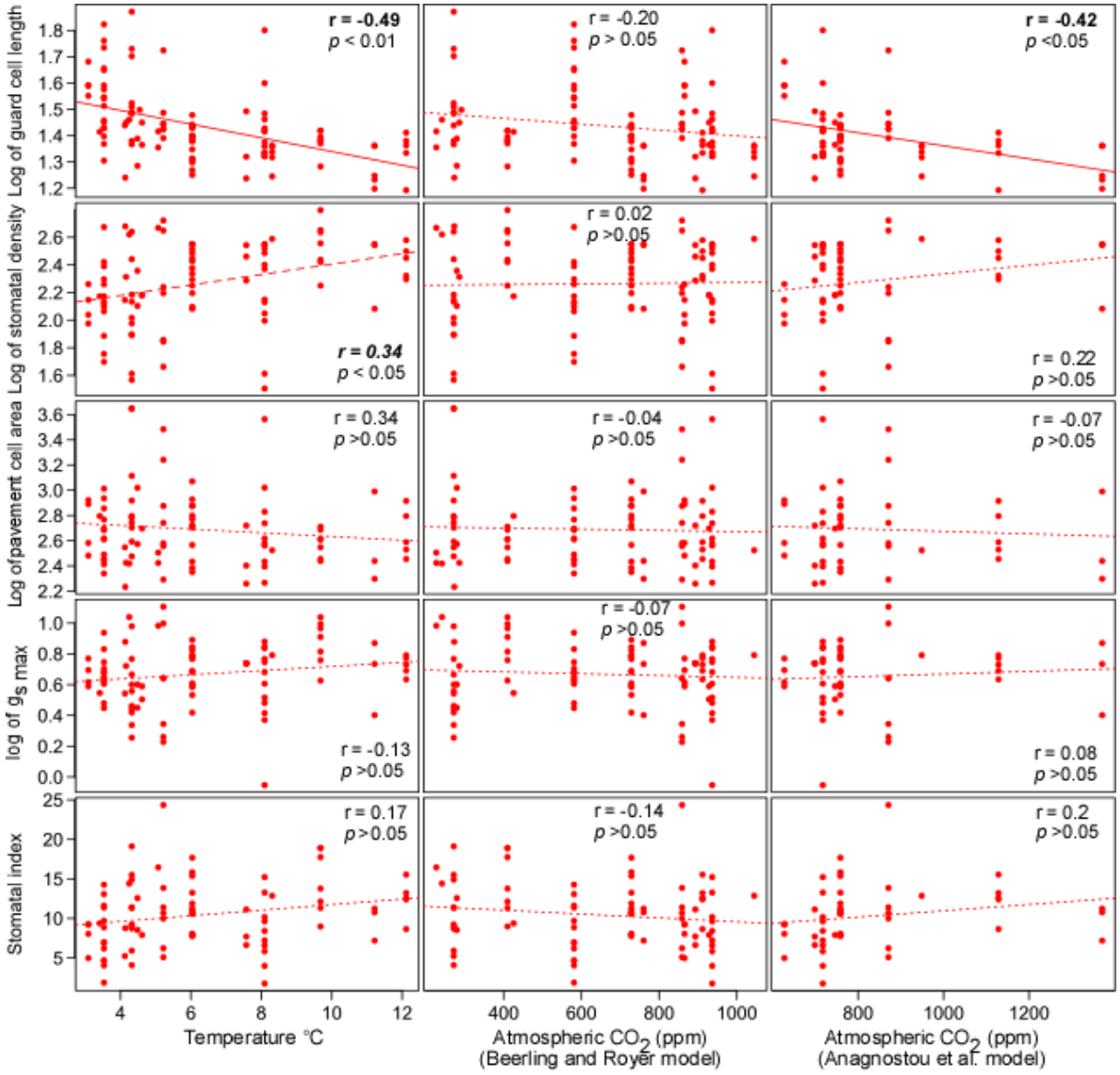

Figure 2. Epidermal traits for fossils versus estimated deep sea temperature [29], and atmospheric CO_2 [17, 18]. Probabilities are based on tests allowing for evolutionary change (electronic supplementary material, figure S2). The estimated probabilities vary with μ/λ, and the value shown reflects all estimates for the given correlation. Solid lines indicate significant linear regressions (p <0.05), dashed line indicates a marginally significant regression (p <0.05 under some modelling parameters), dotted lines indicate non-significant trends.

The multiple regression analysis reinforced these results: the best model for log of guard cell length included temperature and atmospheric CO_2 as estimated by Beerling and Royer [17], with the latter having a positive slope (electronic supplementary material, table S8). The best model for stomatal

density had atmospheric CO_2 as estimated by Anagnostou et al. [18] as the best predictor, but this was a very weak relationship (R^2 = 0.06). The best models for stomatal index, log of pavement cell area and low of theoretical conductance were null models (no predictors).

The correlations among epidermal traits observed on the fossil Proteaceae (figure 3) were consistent with predictions assuming the maintenance of developmental coordination of epidermal cell types through time [13], with positive associations between pavement cell area and both guard cell length and stomatal index and between stomatal index and stomatal density; and negative associations between stomatal density and both pavement cell area and guard cell length. Among age and pooled within age correlations (electronic supplementary material, figure S3) were also consistent with developmental coordination.

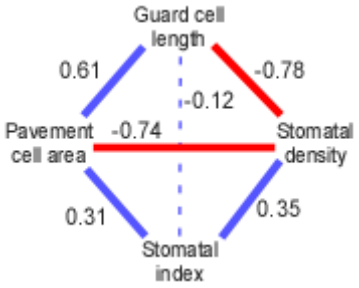

Figure 3. Pearson correlations among epidermal traits observed on fossils. Thickness of lines represents relative strength of correlations. Data excluded Cretaceous fossils, but inclusion of all data produced very similar results. Correlations with $g_{s\,max}$ are not shown because $g_{s\,max}$ is derived from the other traits.

4. Discussion

The systematic changes in guard cell length (and hence stomatal size) through the latest Cretaceous, Paleogene and early Neogene (~70–15 Ma) in Proteaceae are best explained by adaptation to habitat change, as previously argued [15]. Guard cell lengths of fossils in this family are predicted by past global temperatures, but the associations between atmospheric CO_2 and stomatal traits showed trends opposite to those predicted based on conservation of photosynthetic CO_2 inside the leaf [37].

We do not believe that these signals are biased by preservation processes. Although shrinkage of cuticles during fossilisation may affect the size of fossil stomata, such shrinkage is likely to result from changes in chemical composition and should affect both pavement and guard cells more-or-less equally. However, pavement cell area shows patterns that are weaker than and different from those for guard cells (figure 1). Furthermore, systematic temporal trends in the degree of shrinkage appear unlikely because the requirement for rapid preservation of these organically-preserved fossils means that most diagenetic processes likely operated on the leaves soon after deposition.

Changes in atmospheric CO_2 may well have affected guard cell length, stomatal density, stomatal index or maximum stomatal conductance ($g_{s\,max}$) as suggested by broad scale studies showing an association between fossil guard cell size and estimated past CO_2 [5, 12]. However, our results show unexpected relationships between the epidermal traits and estimated trends in palaeo-CO_2 [17, 18] implying that other environmental factors affect stomatal traits. This is supported by evidence that $g_{s\,max}$ is affected by growth form and vegetation structure across species [38]. With regard to our results, standard models for estimating atmospheric CO_2 from stomata (as summarised in [1]) predict CO_2 to be positively associated with guard cell length and negatively associated with stomatal index, stomatal density and $g_{s\,max}$, but our data shows correlations that are either opposite to expectations or near zero (figures 1 & 2). The only hint of an expected relationship with palaeo CO_2 was that the best multiple regression model for log of guard cell length included a positive slope for the Beerling and Royer model [17]) (electronic supplementary material, table S8). None of the best models for more direct potential predictors of palaeo CO_2 (stomatal index, stomatal density or $g_{s\,max}$) showed the expected relationships.

We propose that, given the strong link between vegetation type and stomatal size in extant Proteaceae [15], the strong association between fossil guard cell length and palaeotemperature (figure 1) may result from the association of higher global temperatures with wetter climates and more closed forest through time. Fossil evidence shows that in our study area, relatively open vegetation in the late Cretaceous [39] was replaced by widespread rainforest in the warm, and very wet Early Eocene,

which was then progressively replaced with open vegetation as climates became cooler, drier and more fire prone [40-42]. Similar associations amongst environmental drivers can be observed today, with closed forest being closely linked to warm and wet climates [43, 44]. The functional basis of the link between stomatal size and forest structure is poorly understood, but could be underpinned by differences in environmental productivity, with open vegetation species being found in low nutrient or cold environments in Proteaceae, and closed forest species in more productive environments [15]. Thus smaller, more numerous stomata may reflect shifts to more productive species, as has been observed at long evolutionary timescales [45]

5. Conclusions

Patterns of cell sizes and abundance in the fossils suggest that coordinated development of leaf tissues observed in extant Proteaceae [13] is maintained through evolutionary time. Our results provide fossil evidence to strengthen inferences from extant species [2, 15] that habitat changes associated with climate are major drivers of changes in stomatal size (and other aspects of the epidermis) in Proteaceae. This has significant implications for stomatal proxies for past CO_2. The stomatal proxies for CO_2 mostly consider variation within species through time and the evidence presented here largely reflects variation among species. Although, these species-specific proxies assume that there has been no change in the relationship between CO_2 levels and the relevant stomatal traits, often over tens of millions of years [2], differences in the CO2/stomata relationship can be observed amongst closely related species, implying that evolution and/or aspects of environment other than CO_2 can affect this relationship [2]. The assumption may be particularly problematic for some widely-used palaeo CO_2 proxies, such as the proxy based on *Ginkgo biloba*, which uses extant populations that represent only a tiny proportion of the past range of once widespread species and clades [2]. The results presented here highlight the importance of adaptive evolutionary changes in stomata over an evolutionary timescale. Further refinement of proxies should therefore consider the impact of other environmental effects on stomatal characteristics.

The new approach for testing correlations of fossils with environmental traits developed here adds to the paleoenvironmental toolbox, particularly since we show that failing to allow for evolution increases the rate of false positives in tests of correlations involving fossils, especially in clades with high levels of extinction (electronic supplementary material, table S7 and figure S2).

Data accessibility

Data are in electronic supplementary material, tables S2 and S4. Code is available in electronic supplementary material, table S9, apart from the *fasttree* function, which is available at https://github.com/MichaelWoodhams/stomata. Stomatal measurements: https://doi.org/10.5061/dryad.5qfttdz1p.

Author contributions:

G.J.J., R.J.C., B.R.H., M.D.W. and T.J.B. designed the research. R.J.C., G.J.J, N.J.B. and , M.D.W. performed the research. All authors wrote the paper and gave final approval for publication.

Competing interests

We declare we have no competing interests.

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References

[1] McElwain, J.C. & Steinthorsdottir, M. 2017 Paleoecology, ploidy, paleoatmospheric composition, and developmental biology: A review of the multiple uses of fossil stomata. *Plant Physiology* **174**, 650-664. (doi:10.1104/pp.17.00204).

[2] Jordan, G.J. 2011 A critical framework for the assessment of biological palaeoproxies: Predicting past climate and levels of atmospheric CO_2 from fossil leaves. *New Phytologist* **192**, 29-44. (doi:10.1111/j.1469-8137.2011.03829.x).

[3] Franks, P.J., Royer, D.L., Beerling, D.J., Van De Water, P.K., Cantrill, D.J., Barbour, M.M. & Berry, J.A. 2014 New constraints on atmospheric CO2 concentration for the Phanerozoic. *Geophysical Research Letters* **41**, 4685-4694. (doi:10.1002/2014GL060457).

[4] Roth-Nebelsick, A., Oehm, C., Grein, M., Utescher, T., Kunzmann, L., Friedrich, J.P. & Konrad, W. 2014 Stomatal density and index data of *Platanus neptuni* leaf fossils and their evaluation as a CO_2 proxy for the Oligocene. *Review of Palaeobotany and Palynology* **206**, 1-9. (doi:10.1016/j.revpalbo.2014.03.001).

[5] Franks, P.J. & Beerling, D.J. 2009 Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 10343-10347. (doi:10.1073/pnas.0904209106).

[6] Assouline, S. & Or, D. 2013 Plant water use efficiency over geological time - evolution of leaf stomata configurations affecting plant gas exchange. *PLoS ONE* **8**. (doi:10.1371/journal.pone.0067757).

[7] de Boer, H.J., Price, C.A., Wagner-Cremer, F., Dekker, S.C., Franks, P.J. & Veneklaas, E.J. 2016 Optimal allocation of leaf epidermal area for gas exchange. *New Phytol.* **210**, 1219-1228. (doi:10.1111/nph.13929).

[8] Elliott-Kingston, C., Haworth, M., Yearsley, J.M., Batke, S.P., Lawson, T. & McElwain, J.C. 2016 Does size matter? Atmospheric CO_2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO_2. *Frontiers in Plant Science* **7**. (doi:10.3389/fpls.2016.01253).

[9] Franks, P.J., Freckleton, R.P., Beaulieu, J.M., Leitch, I.J. & Beerling, D.J. 2012 Megacycles of atmospheric carbon dioxide concentration correlate with fossil plant genome size. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367**, 556-564. (doi:10.1098/rstb.2011.0269).

[10] Wong, S.C., Cowan, I.R. & Farquhar, G.D. 1979 Stomatal conductance correlates with photosynthetic capacity. *Nature* **282**, 424–426.

[11] Parlange, J.-Y. & Waggoner, P.E. 1970 Stomatal dimensions and resistance to diffusion. *Plant Physiology* **46**, 337-342.

[12] Franks, P.J. & Beerling, D.J. 2009 CO_2-forced evolution of plant gas exchange capacity and water-use efficiency over the Phanerozoic. *Geobiology* **7**, 227-236. (doi:10.1111/j.1472-4669.2009.00193.x).

[13] Brodribb, T.J., Jordan, G.J. & Carpenter, R.J. 2013 Unified changes in cell size permit coordinated leaf evolution. *New Phytologist* **199**, 559-570. (doi:10.1111/nph.12300).

[14] Franks, P.J., Bonan, G.B., Berry, J.A., Lombardozzi, D.L., Holbrook, N.M., Herold, N. & Oleson, K.W. 2018 Comparing optimal and empirical stomatal conductance models for application in Earth system models. *Global Change Biology* **24**, 5708-5723. (doi:10.1111/gcb.14445).

[15] Jordan, G.J., Carpenter, R.J., Koutoulis, A., Price, A. & Brodribb, T.J. 2015 Environmental adaptation in stomatal size independent of the effects of genome size. *New Phytologist* **205**, 608-617. (doi:10.1111/nph.13076).

[16] Zachos, J., Pagani, H., Sloan, L., Thomas, E. & Billups, K. 2001 Trends, rhythms, and aberrations in global climate 65 Ma to present. *Science* **292**, 686-693.

[17] Beerling, D.J. & Royer, D.L. 2011 Convergent Cenozoic CO_2 history. *Nature Geoscience* **4**, 418-420. (doi:10.1038/ngeo1186).

[18] Anagnostou, E., John, E.H., Edgar, K.M., Foster, G.L., Ridgwell, A., Inglis, G.N., Pancost, R.D., Lunt, D.J. & Pearson, P.N. 2016 Changing atmospheric CO_2 concentration was the primary driver of early Cenozoic climate. *Nature* **533**, 380-384. (doi:10.1038/nature17423).

[19] Byrne, M., Steane, D.A., Joseph, L., Yeates, D.K., Jordan, G.J., Crayn, D., Aplin, K., Cantrill, D.J., Cook, L.G., Crisp, M.D., et al. 2011 Decline of a biome: Evolution, contraction, fragmentation, extinction and invasion of the Australian mesic zone biota. *Journal of Biogeography* **38**, 1635-1656. (doi:10.1111/j.1365-2699.2011.02535.x).

[20] Carpenter, R.J. 2012 Proteaceae leaf fossils: phylogeny, diversity, ecology and austral distributions. *Botanical Review* **78**, 261-287. (doi:10.1007/s12229-012-9099-y).

[21] Felsenstein, J. 1985 Phylogenies and the comparative method. *American Naturalist* **125**, 1-15.

[22] Carpenter, R.J., Hill, R.S. & Jordan, G.J. 2005 Leaf cuticular morphology links Platanaceae and Proteaceae. *International Journal of Plant Sciences* **166**, 843-855. (doi:10.1086/431806).

[23] Burnham, R.J. 1989 Relationships between standing vegetation and leaf litter in a paratropical forest: Implications for paleobotany. *Review of Palaeobotany and Palynology* **58**, 5-32. (doi:10.1016/0034-6667(89)90054-7).

[24] Sauquet, H., Weston, P.H., Anderson, C.L., Barker, N.P., Cantrill, D.J., Mast, A.R. & Savolainen, V. 2009 Contrasted patterns of hyperdiversification in Mediterranean hotspots. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 221-225. (doi:10.1073/pnas.0805607106).

[25] Stadler, T. 2011 Simulating trees with a fixed number of extant species. *Systematic Biology* **60**, 676-684. (doi:10.1093/sysbio/syr029).

[26] Stadler, T. 2019 TreeSim. R package version 2.4.

[27] R Core Team. 2014 *R: A language and environment for statistical computing*, R Foundation for Statistical Computing, Vienna, Austria.

[28] Wood, S. 2018 Package 'mgcv'. R package version 1.8.

[29] Hansen, J., Sato, M., Kharecha, P., Beerling, D., Berner, R., Masson-Delmotte, V., Pagani, M., Raymo, M., Royer, D.L. & Zachos, J.C. 2008 Target atmospheric CO_2: Where should humanity aim? *arXiv preprint arXiv:0804.1126*.

[30] Pennell, M.W., Fitzjohn, R.G., Cornwell, W.K. & Harmon, L.J. 2015 Model adequacy and the macroevolution of angiosperm functional traits. *American Naturalist* **186**, E33-E50. (doi:10.1086/682022).

[31] Hill, R.S., Scriven, L.J. & Jordan, G.J. 1995 The fossil record of Australian Proteaceae. In *Flora of Australia* (ed. P.M. McCarthy), pp. 21–30. Canberra, ACT, Australia, Australian Biological Resources Study.

[32] Harvey, P.H. & Rambaut, A. 1998 Phylogenetic extinction rates and comparative methodology. *Proceedings of the Royal Society B: Biological Sciences* **265**, 1691-1696. (doi:10.1098/rspb.1998.0490).

[33] Pybus, O.G. & Harvey, P.H. 2000 Testing macro-evolutionary models using incomplete molecular phylogenies. *Proceedings of the Royal Society B: Biological Sciences* **267**, 2267-2272. (doi:10.1098/rspb.2000.1278).

[34] Ho, L.S.T., Ane, C., Lachlan, R., Tarpinia, K., Feldman, R., Yu, Q. & Bijl, W.v.d. 2018 phylolm. R package version 2.6.

[35] Manly, B.F.J. 1997 *Randomization, bootstrap and Monte Carlo methods in biology*. London, UK, Chapman and Hall/CRC; 424 p.

[36] Bartoń, K. 2019 MuMIn. R package version 1.43.6.

[37] Franks, P.J., Leitch, I.J., Ruszala, E.M., Hetherington, A.M. & Beerling, D.J. 2012 Physiological framework for adaptation of stomata to CO_2 from glacial to future concentrations. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367**, 537-546. (doi:10.1098/rstb.2011.0270).

[38] Reichgelt, T. & D'Andrea, W.J. 2019 Plant carbon assimilation rates in atmospheric CO2 reconstructions. *New Phytologist* **223**, 1844-1855. (doi:10.1111/nph.15914).

[39] Carpenter, R.J., Macphail, M.K., Jordan, G.J. & Hill, R.S. 2015 Fossil evidence for open, Proteaceae-dominated heathlands and fire in the Late Cretaceous of Australia. *Am. J. Bot.* **102**, 2092-2107. (doi:10.3732/ajb.1500343).

[40] Hill, R.S., Truswell, E.M., McLoughlin, S. & Dettmann, M.E. 1999 Evolution of the Australian flora: fossil evidence. In *Flora of Australia. Vol. 1, 2nd Edn.* (ed. A.E. Orchard). Melbourne, ABRS/CSIRO.

[41] Dunn, R.E., Strömberg, C.A.E., Madden, R.H., Kohn, M.J. & Carlini, A.A. 2015 Linked canopy, climate, and faunal change in the Cenozoic of Patagonia. *Science* **347**, 258-261. (doi:10.1126/science.1260947).

[42] Lee, D.E., Lee, W.G., Jordan, G.J. & Barreda, V.D. 2016 The Cenozoic history of New Zealand temperate rainforests: comparisons with southern Australia and South America. *New Zealand Journal of Botany* **54**, 100-127. (doi:10.1080/0028825X.2016.1144623).

[43] Bond, W.J. 2005 Large parts of the world are brown or black: A different view on the 'Green World' hypothesis. *Journal of Vegetation Science* **16**, 261-266. (doi:10.1111/j.1654-1103.2005.tb02364.x).

[44] Polis, G.A. 1999 Why are parts of the world green? Multiple factors control productivity and the distribution of biomass. *Oikos* **86**, 3-15. (doi:10.2307/3546565).

[45] Simonin, K.A. & Roddy, A.B. 2018 Genome downsizing, physiological novelty, and the global dominance of flowering plants. *PLoS Biology* **16**. (doi:10.1371/journal.pbio.2003706).