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PINNULE AND STOMATAL SIZE AND STOMATAL DENSITY OF LIVING AND FOSSIL *BOWENIA* AND *EOBOWENIA* SPECIMENS GIVE INSIGHT INTO PHYSIOLOGY DURING CRETACEOUS AND EOCENE PALEOCLIMATES

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Premise of research. This study compares the pinnule morphology of three fossil *Bowenia* species, one *Eobowenia* (fossil) species, and several *Bowenia* fossil pinnule fragments with the two extant *Bowenia* species.

Methodology. Pinnule area, stomatal density, and size have been measured on fossil and extant specimens. Measures have been correlated with solar radiation and temperature to ascertain any correlations. Environment and climate variables have been chosen, as they have changed since the Eocene and Lower Cretaceous when the Australian and South American fossils were growing.

Pivotal results. Two of the fossil *Bowenia* species and *Eobowenia* have significantly smaller pinnules than the living species, and all the fossils have relatively small and sparse stomata compared with the living species. Extant *Bowenia* pinnule area is positively correlated with daily radiation and temperature.

Conclusions. Those fossil species with small pinnules likely lived in relatively dark environments where small pinnules aided the capture of sunflecks and/or facilitated light capture. The smaller and sparser stomata may have limited the potential sites for fungal invasion in the extremely humid growth conditions without hampering overall growth rates in the prevailing very high atmospheric CO₂ levels.

Keywords: pinnule area, stomatal size, cycad, paleobotany, stomatal density, Australian cycad, South American cycad, Eocene, Cretaceous.

Online enhancements: appendix tables.

Introduction

Stomatal size and density are important leaf traits that determine the maximum bounds of leaf gas exchange. While stomata open and close in response to diurnal changes in environmental factors such as light, temperature, and water availability (Prior et al. 1997; Matthews et al. 2018), stomatal morphology varies over longer time scales in response to climate and other environmental variation (Franks and Beerling 2009; Hill et al. 2015). The same is true for leaf area, since the climate influences the leaf boundary layer thickness and photosynthetic area (Wright et al. 2017). This has been widely studied, and stomatal size and density have been shown to respond to humidity (El-Sharkawy et al. 1985; Gislérød and Nelson 1989), temperature (Frank et al. 1973; Limin et al. 2007), atmospheric CO₂ concentration (Woodward 1987; Beerling and Kelly 1997; Bettarini et al. 1998;

Miller-Rushing et al. 2009), water availability (Gindel 1969; Limin et al. 2007), and light (Gay and Hurd 1975; Lake et al. 2001). The response of stomatal morphology and leaf area of fossil plants to climate can be quantified by comparing them with related extant species.

Bowenia Hook. ex Hook.f. is an endemic Australian cycad genus in the family Zamiaceae that contains two extant species in northeastern Australia. The recently described *Eobowenia incrassata* (S.Archang.) M.Coiro et C.Pott comb. nov. from the Aptian of Argentina (Coiro and Pott 2017) is included in the analysis, since it is regarded as the nearest fossil relative of *Bowenia*. Hill (1978) described two macrofossil species of *Bowenia*, based on pinnule remains, from Eocene sediments in southeastern Australia, and recently Hill et al. (2018) reviewed the macrofossil record of *Bowenia* and described a new Early Eocene species from Tasmania. Hill et al. (2018) noted that the three described fossil species had fewer and smaller stomata than those of the extant species, and two of the three fossil species had smaller pinnules than those of the extant species. The purpose of this article is to examine these differences in greater detail and in the context of the growth conditions that prevailed

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in southeastern Australia during the Eocene and in southern South America during the Lower Cretaceous.

Franks and Beerling (2009) showed that, in fossilized cycads, stomatal size varied thirteenfold and stomatal density changed eightfold among species growing between 200 and 40 Ma. These changes were probably a response to changes in environmental conditions. This makes the comparison of the stomatal traits of Australian *Bowenia* and South American *Eobowenia* with the extant *Bowenia* species very important, given the major environmental changes in Australia and globally during the last ~125 Myr. The overall vegetation and habitat at each of the sites that contained the fossil species considered here is understood to varying degrees, but in no case is this understanding close to complete, so information gathered from these fossils has broad impact in assessing how these plants coped in such different environments.

The preservation of most of the fossils considered here allowed a detailed examination of the pinnule area and stomatal density and size. Thus, an assessment of the environments inhabited by these fossil species has been conducted. We investigated relationships between stomatal density, size, and pinnule area with reconstructed changes in light, temperature, rainfall, humidity, and atmospheric CO₂ levels. We compared these traits among the fossil species and the two extant species of *Bowenia* and *Eobowenia* using fossils, herbarium specimens, and contemporary material collected in the field. The fossil *Bowenia* and *E. incrassata* specimens are compared with the extant *Bowenia* species.

Material and Methods

Extant Specimens

Extant *Bowenia* is an understory plant with a reduced stem and usually a single whorl of leaves. Five *Bowenia spectabilis* (Hook.f.) specimens were collected from Butchers Creek on the Atherton Tablelands in Queensland (lat. 17°32'S, long. 145°69'E) in April 2012. Thirty-five more *B. spectabilis* and 13 *Bowenia serrulata* (W.Bull) Chamb. specimens were supplied by the Queensland Herbarium, the National Herbarium of Victoria, and the Australian Tropical Herbarium, spanning 115 yr from 1891 to 2006 for *B. serrulata* and 147 yr, from 1865 to 2012 for *B. spectabilis*. Specimen details are available in tables A1 (*B. spectabilis*) and A2 (tables A1, A2, B1, C1 are available online) (*B. serrulata*). Examples of specimen cuticle images are in figure 2.

Preparation of Cuticles

Cut from the margin were 1 cm² pieces of pinnule. Pinnule pieces were placed into test tubes and covered in 80% ethanol v/v overnight. The ethanol was then replaced with a 2:1 solution of 35% w/v hydrogen peroxide and 80% ethanol v/v and gently heated until the pinnule pieces turned translucent. The pinnule pieces were then rinsed with reverse-osmosis (RO) water and placed under a dissecting microscope so that debris could be brushed away from the cuticle, which was then rinsed with RO water. Cuticles were then stained with crystal violet and mounted on slides in phenol glycerine jelly. All cuticle slides

prepared for this project are housed at the University of Adelaide.

Cretaceous Climate: South America

The Anfiteatro de Ticó Formation is located in Patagonia, Argentina (Coiro and Pott 2017). At the time of fossil deposition, the Aptian, the site was at an extremely high southern latitude of more than 60°S (Archangelsky and Archangelsky 2013).

The region had a rich diversity of cycads (Archangelsky 2001; Del Fueyo 2007), including xeromorphic species (Villar de Seoane 2005). However, the characters interpreted as xeromorphic by these authors may also be interpreted as adaptation to ash fall from surrounding volcanoes. Volcanism is stressful for plants, and many cycads had thick cuticles, papillae, and stomatal wax plugs to cope with these conditions (Haworth and McElwain 2008; Passalia et al. 2010). Because of the high volcanism, the sun was probably regularly blocked by ash fall and thus *Eobowenia incrassata* would have often persisted in low light conditions. This may have also created an environment of acid precipitation and burial following volcanic events (Limarino et al. 2012). During the Aptian, conditions in the area were warm with a dry season including intermittent humidity caused by volcanism (Limarino et al. 2012). Atmospheric CO₂ conditions were high, with some estimates up to 2500 ppm, although many estimates of Aptian CO₂ are ~1100 ppm (Wang et al. 2014).

Eocene Climate: Australia

The Australian macrofossil taxa were recovered from Campanian-Maastrichtian and Early and Middle Eocene sediments. During the Eocene, Australia was beginning to separate from Antarctica and was ~20° farther south than at present. However, the prevailing climate at that latitude was very different than it is today. A combination of several features (ocean circulation patterns, atmospheric composition, and CO₂ levels, and the general configuration of the continents) meant that the temperatures during the first half of the Eocene were much higher than they are today, and this is particularly notable at high southern latitudes. Carpenter et al. (2012) used three proxies to estimate that the Early Eocene site in Tasmania that contains one of the fossil *Bowenia* species (*Bowenia johnsonii*; Hill et al. 2018) had a near-tropical mean annual temperature estimate of ~24°C. Despite the extremely high southern latitude of Tasmania at the time (see fig. 2), McGowran and Hill (2015) noted that the so-called greenhouse-to-icehouse transition has included the Early Eocene climatic optimum, which was the warmest time for 85 Myr. This truncated sharply about 50 Ma, followed by the Middle Eocene climatic optimum and successive shifts in temperature through to the present day. The extent of these Eocene warm periods was such that there is no evidence of ice in Antarctica in the Late Paleocene and Early Eocene, and this remained the case until the early Late Eocene (Francis et al. 2008).

Australia has been moving northward relatively rapidly since its complete separation from Antarctica about 33.5 Ma (Cande and Stock 2013), meaning that all fossil locations were significantly farther south when the plants that produced these fossils

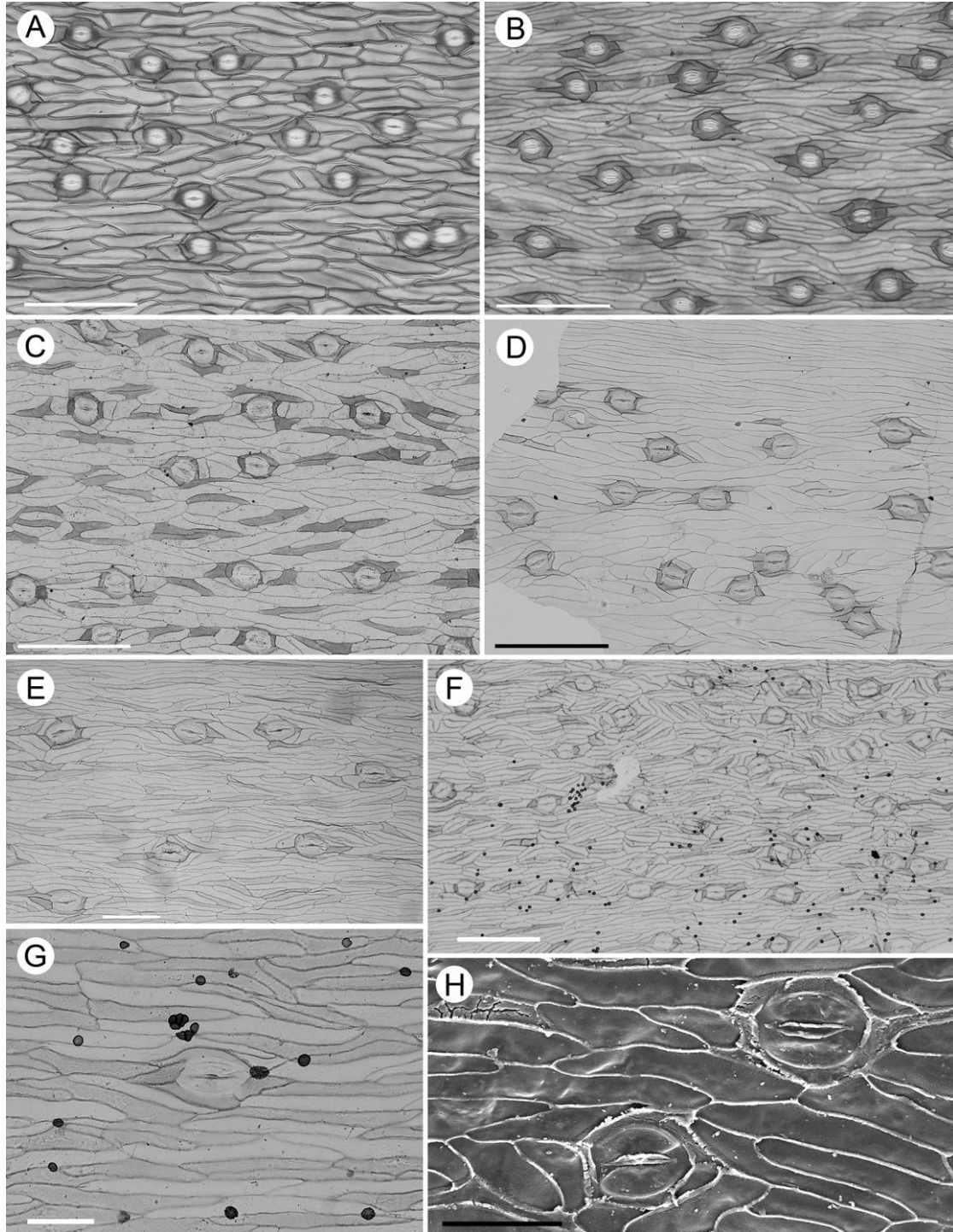


Fig. 1 Cuticle images of living and fossil *Bowenia* species (A–G, light micrographs; H, scanning electron micrograph). Extant species *Bowenia serrulata* (A) and *Bowenia spectabilis* (B) showing the abaxial surface with several stomata present. C, Early Eocene *Bowenia johnstonii* from Lowana Road, showing the probable abaxial surface. D, Late Eocene *Bowenia* sp. from the Rundle locality, showing the probable abaxial surface. E, Late Middle Eocene *Bowenia eocenica* from the Anglesea locality, showing the probable abaxial surface. F, Probable abaxial surface of *B. eocenica* from Anglesea, showing a high density of fungal epiphyllous germlings (sensu Lange 1978). G, A single stoma of *B. eocenica* from Anglesea, showing several epiphyllous germlings attached to the cuticle surface. H, Inner cuticular surface of *B. johnstonii* from Lowana Road, showing the clearly defined stomatal apparatus and the relatively thin cuticular layer between epidermal cells. Scale bars = 200 μm (A–D, F), 100 μm (E), and 50 μm (G, H).

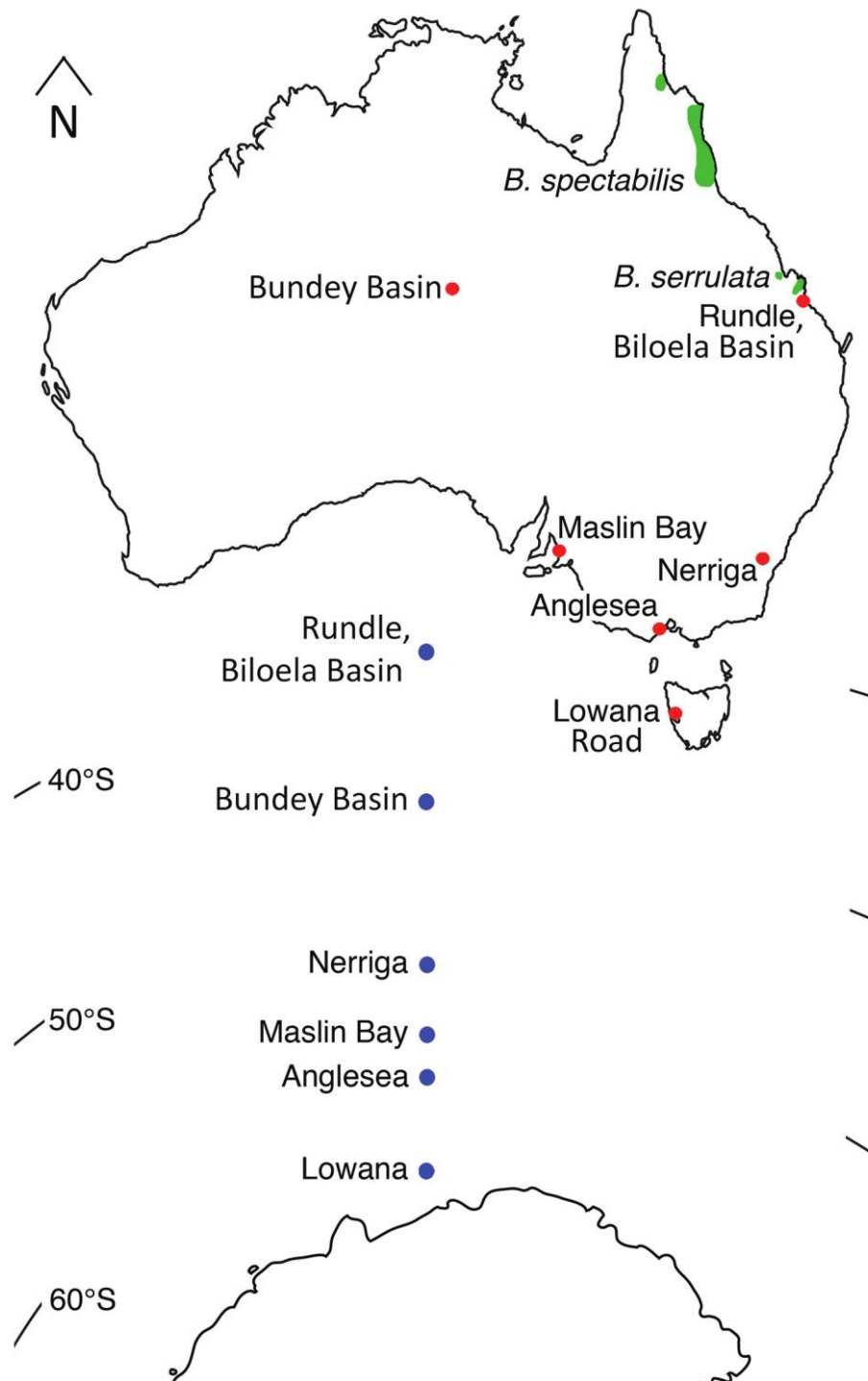


Fig. 2 Map of Australia showing the distribution of the living *Bowenia* species (green), fossil localities considered in detail in this study (red), and approximate palaeolatitude of the fossil localities (as stated by Wilford and Brown 1994) relative to southern Australia and Antarctica as they are placed today (blue).

were growing. The palaeolatitude of each fossil location can be estimated, and from that estimate the mean daily irradiance can be calculated, based on modern levels. However, for at least the Eocene localities, there is a high probability that heavy cloud cover was much more prevalent than it is now (Sloan and Rea

1996), so these irradiance estimates should be regarded as maximum possible values. It should also be noted that small understory plants like *Bowenia* (assuming the fossil species had a similar habit to the two extant species) would have received much less of this radiation than canopy plants. Mean daily irradiance

of the Rundle fossil location (most northerly; fig. 2) site during the Late Eocene is estimated to be $446 \pm 20 \text{ W m}^{-2}$ (Laskar et al. 2004), while the irradiance for the most southerly fossil site, Lowana Road, was estimated to be $425 \pm 19 \text{ W m}^{-2}$ (for insolation calculation information, see table 1; Laskar et al. 2004). The current mean irradiance of the sites where extant *Bowenia* grows is $\sim 436.8 \text{ W m}^{-2}$ for *B. serrulata* and 441.4 W m^{-2} for *B. spectabilis* (Shellito et al. 2003; Laskar et al. 2004). It is also important to note that at the high latitudes where the plants that produced the fossils were growing, the sun has a daily track that remains relatively close to the horizon. Hence, it must have been particularly challenging for understory plants to receive adequate sunlight for growth, since shading from surrounding vegetation and topography is likely to have been high.

Other important plant growth factors were probably water availability and atmospheric CO_2 , both of which varied during the past and up to the present day. There is abundant evidence that Australia had very high humidity during the Early-Middle Eocene (Martin 2006) and perhaps levels not recorded anywhere on Earth today (Hill 2004). But by the end of the Late Eocene, the climate had started to dry, for a variety of reasons, including Australia's separation from Antarctica and the initiation of the circum-Antarctic Ocean Current (Hill 2004) and declining atmospheric CO_2 levels (Anagnostou et al. 2016). Atmospheric CO_2 levels during the Late Campanian-Maastrichtian were ~ 800 ppm (Wang et al. 2014). During the Early Eocene CO_2 levels were possibly up to 1400 ppm, but by the beginning of the Late Eocene they had dropped to ~ 770 ppm (Anagnostou et al. 2016). Carbon dioxide levels continued to fall to less than 300 ppm in the Holocene, until the commencement of the Industrial Revolution, and the level is now above 400 ppm and rising (Tans and Keeling 2017).

Extant Climate

Environmental conditions for locations of the *B. spectabilis* and *B. serrulata* collections were obtained from the Atlas of Living Australia (<http://spatial.ala.org.au/#>). The data collected from this website are available in table B1. All definitions of environmental variables pertaining to these collections were obtained from the Atlas of Living Australia (<http://spatial.ala.org.au/#>) and are defined as follows: (1) minimum month evaporation (mm; minimum evaporation of any monthly evaporation), (2) minimum month vapor pressure deficit (KPa), (3) precipitation during the wettest month (mm; maximum rainfall of any

monthly rainfall), (4) radiation during the lowest period of the day ($\text{MJ m}^{-2} \text{ d}^{-1}$; Bio22), and (5) maximum temperature ($^{\circ}\text{C}$; maximum temperature of any monthly maximum temperature). Atmospheric CO_2 when the contemporary *Bowenia* specimens were collected in 2012 was 397 ppm (Tans and Keeling 2017), which is an increase of ~ 100 ppm since the beginning of the previous century (Etheridge et al. 1998), when the earliest herbarium specimens were collected.

Fossil Pinnules and Cuticles

All fossil specimens had been prepared previously and were either retrieved from the University of Adelaide's David T. Blackburn Palaeobotany Collection, the South Australian State Herbarium, the Melbourne Museum, the University of Tasmania, or publication images. To maximize fossil data, any cuticle or pinnule remains that can be assigned to *Bowenia* have been included. When they have not been allocated to species, they will be referred to by their site names. Bunday Basin *Bowenia* has been described—although not formally—as indistinguishable from extant *Bowenia* (Carpenter et al. 2015). The Rundle/Biloela Basin sites have also been found to contain cuticles of *Bowenia* (Macphail et al. 2014); these are also not described (Foster and Harris 1981; Rowett 1988; Macphail et al. 2014). The final site containing *Bowenia* not formally described is Maslin Bay (McGowran et al. 1970). *Eobowenia* has been included because it is a sister group to *Bowenia*, and the two genera have a Gondwanan distribution (Coiro and Pott 2017). The general morphology of the fossils and their identification is found in Hill (1978), Coiro and Pott (2017), and Hill et al. (2018). For further details, see table C1.

Measurements

Cuticles were examined with an Olympus AX70 microscope, and cuticle photos were taken using the AnalySIS (ver. 6.0.6001 Service Pack 1 Build 6001, Acer, Australia) software. Stomatal density (stomata mm^{-2}) was measured on both the fossil and extant pinnule cuticles by drawing a box measuring $400 \times 400 \mu\text{m}$ on the cuticle photo and counting the number of stomata per box. Stomatal density was determined as the number of stomata per mm^2 . Stomatal size (μm^2) was calculated as the product of the length and width of between two and six guard cell pairs per piece of cuticle. Replication varied depending on availability of pinnules (*B. johnsonii*, $n = 5$; *Bowenia eocenica* R.S. Hill, $n = 3$; Rundle/Biloela Basin, $n = 2$; *Bowenia papillosa* R.S. Hill,

Table 1

Location and Age of the Fossil Sites

Site	Location (lat., long.)	Age (mya)	References
Lowana Road	42°11'S, 145°22'E	Early Eocene 56–47.8	Carpenter et al. 2012
Nerriga	35°07'S, 150°05'E	Early-Middle Eocene boundary 47.8	Wellman and McDougall 1974; Truswell and Owen 1988
Maslin Bay	35°13'S, 138°29'E	Early Middle Eocene 56–38	McGowran et al. 1970
Anglesea	38°25'S, 144°11'E	Late Middle Eocene 47.8–33.9	Christophel et al. 1987
Rundle/Biloela Basin	23°40'S, 151°10'E	Late Eocene 38–33.9	Foster and Harris 1981; Rowett 1988; Macphail et al. 2014
Bunday Basin	~22°39'S, 135°15'E	Late Campanian-Maastrichtian	Carpenter et al. 2015
Estancia Bajo Grande, Argentina	46°57'S, 69°17'W	Lower Cretaceous (Aptian)	Coiro and Pott 2017

$n = 1$; and Maslin Bay, $n = 1$; Bunday Basin, $n = 1$; *Eobowenia incrassata*, $n = 1$; *B. serrulata*, $n = 13$; *B. spectabilis*, $n = 40$). Stomatal index was difficult to measure consistently due to the distribution of the stomata in bands. This made measurements on fossil material difficult, as small pieces of cuticle often yielded less than one band; thus, stomatal index is not considered here.

Whole-pinnule ($n = 19$; table C1) images of herbarium, field-collected, and fossil specimens were captured using a CanoScan Toolbox version 4.9.3.2toolpak (X for Mac OSX) flatbed scanner. Pinnule area (mm^2) was determined with ImageJ 1.44p (Rasband 1997–2014). Whole-pinnule fossil images were only available for *B. eocenica*, *B. papillosa*, and *B. johnsonii*, and even then extrapolation was sometimes required to determine pinnule area (see images in Hill et al. 2018).

Shapiro-Wilk tests and multiple linear models with analysis of variance run on modern data used RStudio (RStudio Team 2015). Subsequently, the presence of interactions between variables that had significant effects on pinnule anatomy or size were tested. Due to the nature of the fossil material there was a very low rate of replication and, as such, a random mixed effects model was inappropriate. We present these data at face value, although we are aware that higher replication would improve the robustness of the work.

Results

Stomatal density and size did not correlate with each other for the entire 125 Myr time frame ($R^2 = 0.02$, $P = 0.09$; fig. 3). Stomatal size of all fossil taxa is significantly smaller than

their extant counterparts, except for *Bowenia papillosa* and the Maslin Bay specimen, which are not significantly different than either the fossil or extant species (fig. 4), although they overlapped at the small size range of the extant species. Stomatal density of four of the five measurable fossil species was not significantly different from either the other fossil or extant species (fig. 5). *Bowenia johnsonii* had significantly fewer stomata per unit area than the extant species. Stomatal data did not correlate with any environmental variables. Pinnule area of extant specimens correlated with low periods of irradiation ($R^2 = 0.12$, $P < 0.0001$; fig. 6) and maximum temperatures ($R^2 = 0.13$, $P < 0.0001$; fig. 7). There were no significant correlations between stomatal size and density with any climate parameters tested (table B1). Pinnule area also did not significantly correlate with these variables.

Figure 8 is an illustration of pinnule area of all known species of *Bowenia* (note mean values for these measures in table 2). The area of the fossil *Bowenia eocenica* and *Eobowenia* pinnules is significantly smaller than that of the extant pinnules. The pinnule area of *B. papillosa* is not significantly smaller than one extant species, *Bowenia serrulata*, but it is also not significantly different from *B. eocenica* and *Eobowenia incrassata*. *Bowenia johnsonii* pinnule area is significantly larger than that of *B. eocenica* and *E. incrassata* and not significantly different in size from one extant species, *B. serrulata*. Extant *Bowenia spectabilis* has significantly larger pinnules than any others measured.

When fossil and extant pinnules are grouped together, their characters correlate to the large-scale CO_2 change that has occurred between the Cretaceous and the present (fig. 9).

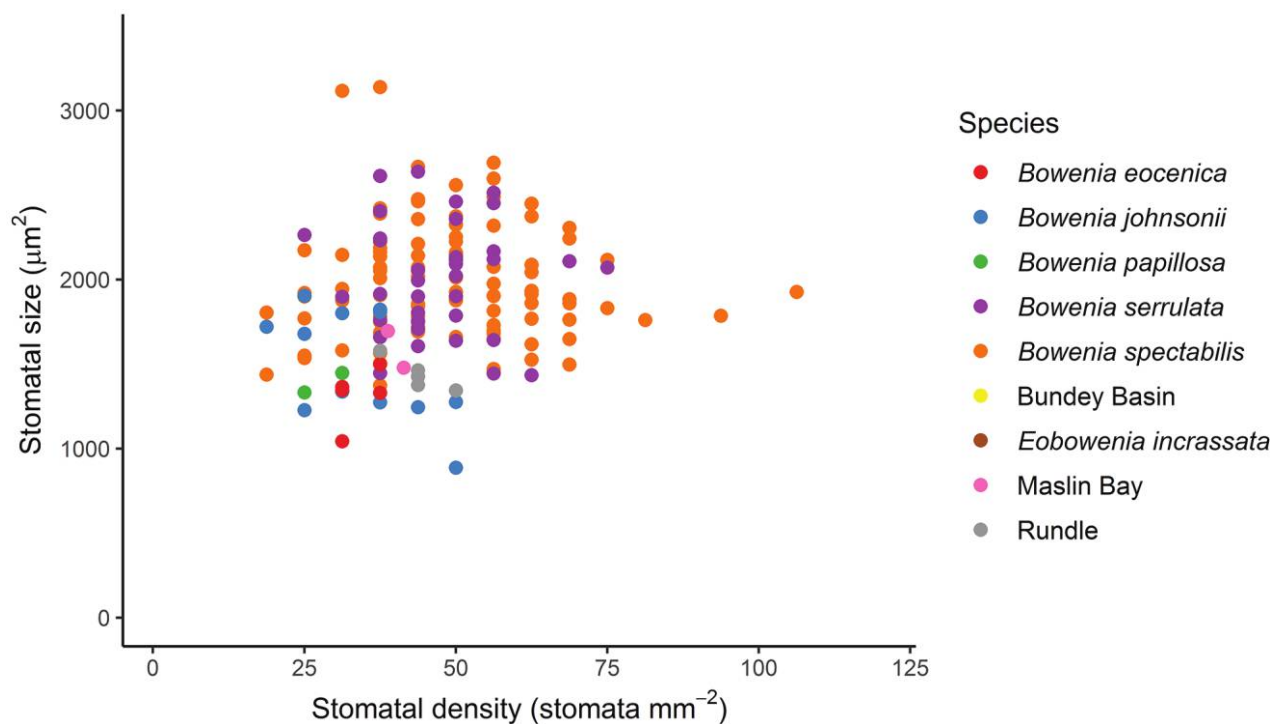


Fig. 3 Stomatal density and size for pinnules of the six Australian fossils, one South American fossil, and two extant species used in this study ($R^2 = 0.02$, $P = 0.09$).

Discussion

The fossil *Bowenia* and *Eobowenia* specimens considered here tended to have fewer, smaller stomata and smaller pinnules than extant species. While for some taxa this was based on only one or a few specimens, it was relatively consistent and where more specimens were available (*Bowenia eocenica* and *Bowenia johnsonii*), although *B. johnsonii* had significantly larger pinnules. The range of stomatal density and size reported in this study (fig. 3) is similar to that found for cycads in other studies, but it is considerably higher than in most angiosperms (e.g., Franks et al. 2009; Drake et al. 2013). Haworth et al. (2011) reported a range of stomatal densities for six cycad species of 30–80 stomata mm^{-2} , whereas the results of this study found stomatal density of *Bowenia* species to be 18–107 stomata mm^{-2} . This is a much larger range than that found by Haworth et al. (2011), and this large range is due to changes in one of the extant species, *Bowenia spectabilis*. All other *Bowenia* and *Eobowenia* species had a range of between 3 and 50 stomata mm^{-2} . *Bowenia spectabilis* occupies a much larger environmental range than any other species tested here (fig. 2) and thus has a larger range of pinnule morphology. Cycads growing during the Phanerozoic have been shown to have a stomatal size of up to 3000 μm^2 (Franks and Beerling 2009), and extant *Bowenia* species can match this at the high end of their range (fig. 3).

The major environmental differences between the extant and fossil environments are irradiance, atmospheric CO_2 , tempera-

ture, relative humidity, and ash fall. The results suggest that pinnule size in extant species is positively correlated with radiation (fig. 6) and temperature (fig. 7); and these are potentially additive effects on pinnule size. Small pinnules are advantageous in shaded environments where light is both low and unpredictable, because they reduce the effects of self-shading (Falster and Westoby 2003) and thus increase the potential to capture sunflecks (Percy and Yang 1996). This may be the case for the fossil *Bowenia* (except *B. johnsonii*) and *Eobowenia*, as they tend to be smaller than the extant species (fig. 8).

The relatively small size of the pinnules at Anglesea, Nerriga, and the Anfiteatro de Ticó Formation (named *B. eocenica*, *B. papillosa*, and *E. incrassata*, respectively) requires careful consideration. Both Australian sites, and especially Anglesea, are characterized by a high angiosperm species diversity and relatively large leaves (Christophel 1980, 1984; Basinger and Christophel 1985; Christophel and Lys 1986; Christophel et al. 1987; Hill and Christophel 1988; Carpenter et al. 2016), both of which have been taken to indicate vegetation with relatively tropical affinities (Christophel et al. 1987), despite the very high latitudes at which they grew. The Anfiteatro de Ticó Formation had a contrasting environment during the Aptian, with low angiosperm and high gymnosperm diversity, and angiosperms present were nymphaeaphyllous (Passalia et al. 2014). Nymphaeaphyllous taxa are the most basal angiosperms with large, rounded leaves similar to those of floating plants (Krassilov 1977). Volcanism dominated the open landscape (Del Fueyo 2007) leading

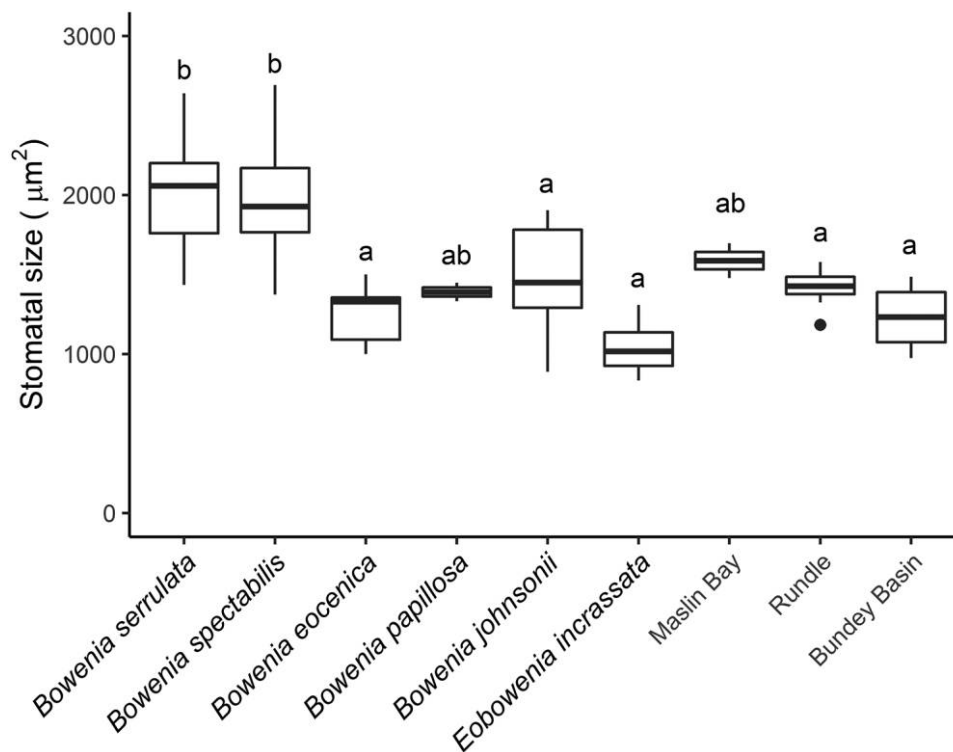


Fig. 4 Boxplots of stomatal size for all known *Bowenia* and *Eobowenia* species. The top and bottom hinges are the 25th and 75th percentiles, respectively. The top and bottom whiskers end at the highest or lowest values, no larger than a multiple of 1.5 times the interquartile range. Data points extending beyond the whiskers are outliers. Letters indicate significant differences.

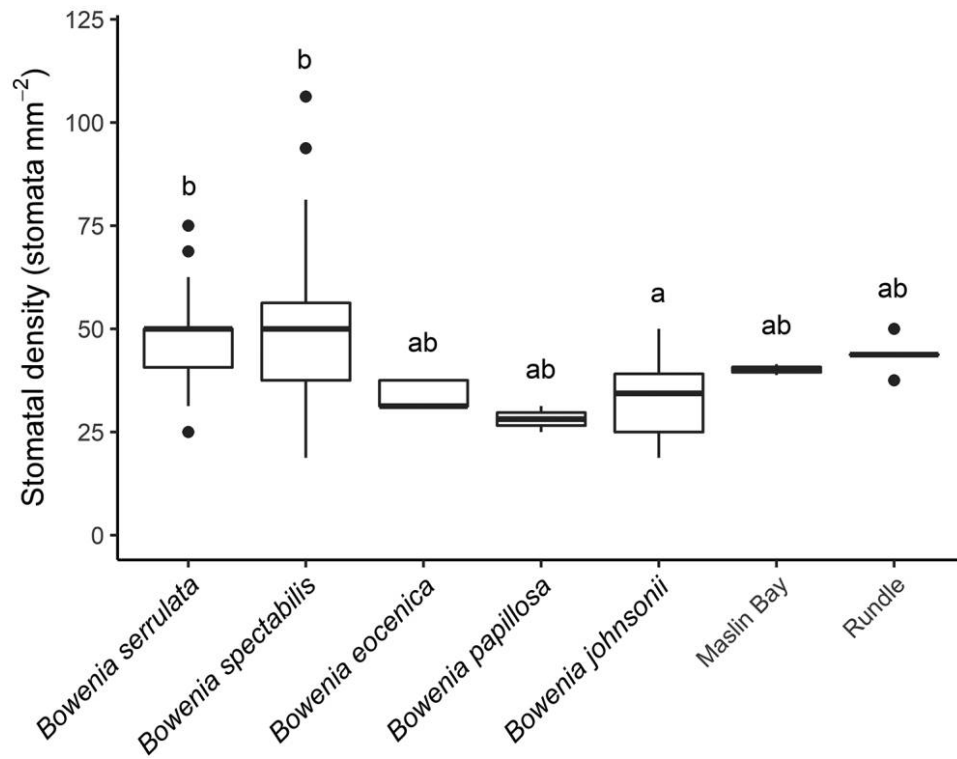


Fig. 5 Boxplots of stomatal density for all measurable known *Bowenia* species. The top and bottom hinges are the 25th and 75th percentiles, respectively. The top and bottom whiskers end at the highest or lowest values no larger than a multiple of 1.5 times the interquartile range. Data points extending beyond the whiskers are outliers. Letters indicate significant differences.

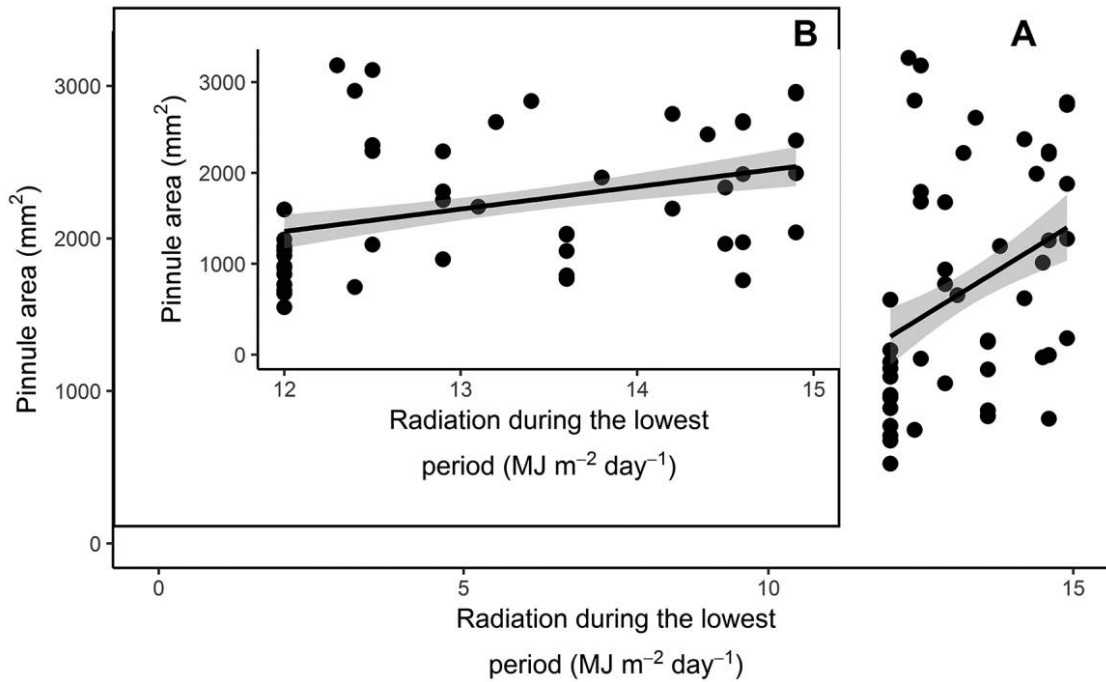


Fig. 6 Relationship between radiation during the lowest period of the day and pinnule area for extant *Bowenia* species ($R^2 = 0.12$, $P < 0.0001$). A, Entire range of radiation. B, Magnified to a $3 \text{ MJ m}^{-2} \text{ d}^{-1}$ span.

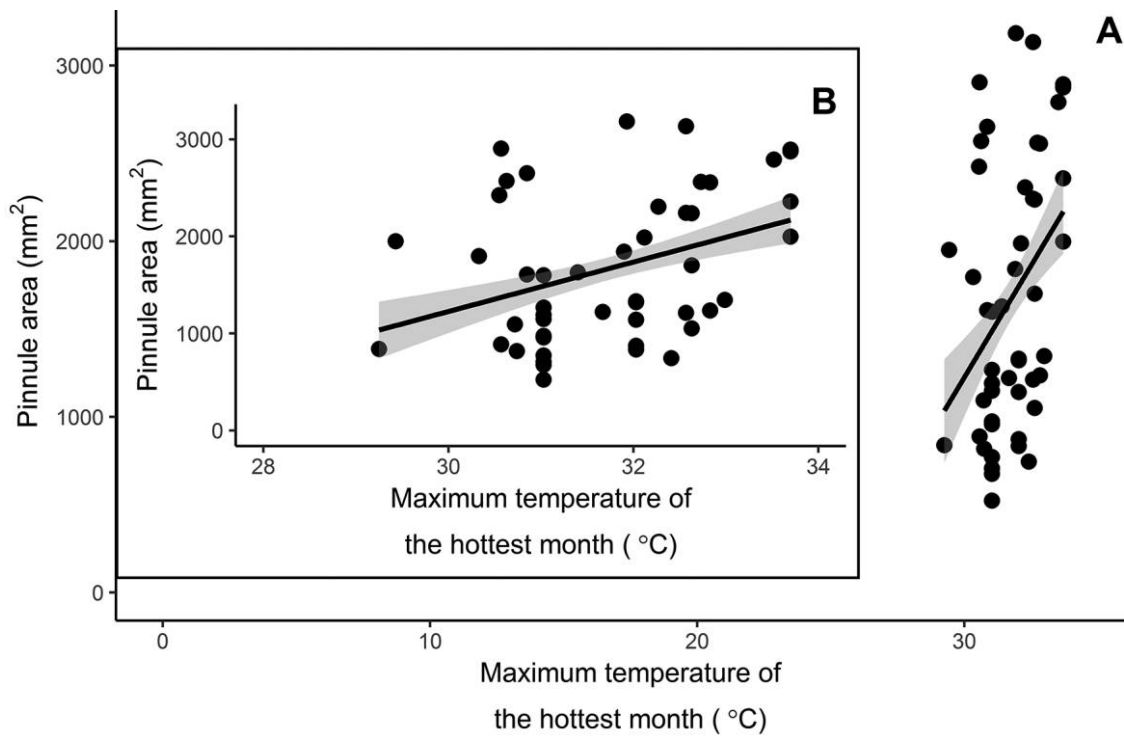


Fig. 7 Relationship between maximum temperature of the hottest month and pinnule area for the extant *Bowenia* species ($R^2 = 0.13$, $P < 0.0001$). A, Entire temperature range. B, Magnified to a 6°C span.

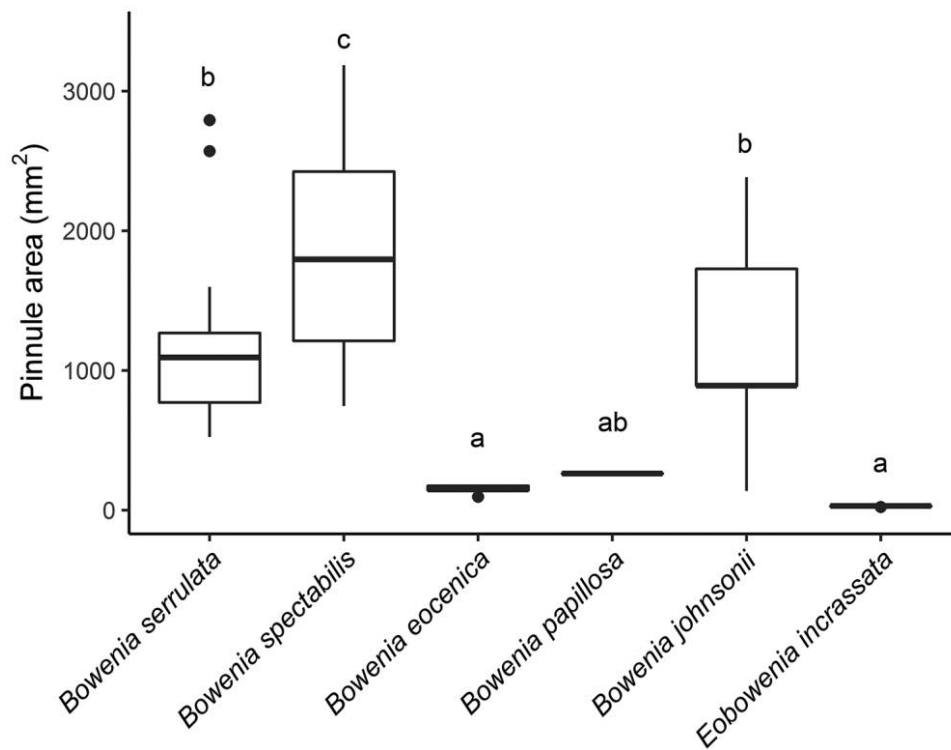


Fig. 8 Boxplots of pinnule area for the two extant and three of the fossil *Bowenia* species and for *Eobowenia*. Letters indicate significant differences.

Table 2
***Bowenia* and *Eobowenia* Species Measurement Means, Standard Errors, and Minimum and Maximum Values for Stomatal Density, Stomatal Size, and Pinnule Area**

Site and species	Stomatal density (stomata mm ⁻²)			Stomatal size (μm ²)			Pinnule area (mm ²)					
	Mean	SE	Minimum value	Maximum value	Mean	SE	Minimum value	Maximum value	Mean	SE	Minimum value	Maximum value
<i>Bowenia spectabilis</i>	49	1.5	18	107	2002	32.9	1374.3	3138.5	1826	72.4	745	3184
<i>Bowenia serrulata</i>	48	1.6	25	75	2009.6	51.4	1434.6	2638.4	1251.4	108.6	523.3	2791.6
<i>Bowenia eocenica</i>	34	1.5	31	38	1245.9	70.6	999.2	1500.1	152.6	8	94.6	179
<i>Bowenia papillosa</i>	29	3.1	25	32	1390	58	1331.9	1448	262.4	0	262.4	262.4
<i>Bowenia johnsonii</i>	35	2.9	19	50	1052.7	67.3	887.7	1903.9	1224.5	177.703	138	2382.3
Maslin Bay <i>Bowenia</i> Rundle/Biloela Basin	40	1.3	39	42	1587.2	108.7	1478.5	1695.9	na	na	na	na
<i>Bowenia</i> Bundey Basin	44	1.6	38	50	1421.4	28.6	1182.8	1578.9	na	na	na	na
<i>Bowenia</i> <i>Eobowenia incrassata</i>	na	na	na	na	1231.3	116	974.5	1485.4	na	na	na	na
<i>Eobowenia incrassata</i>	na	na	na	na	1044	101.3	833.8	1038.6	28.6	1.3	21.8	32.5

Note. na = not applicable.

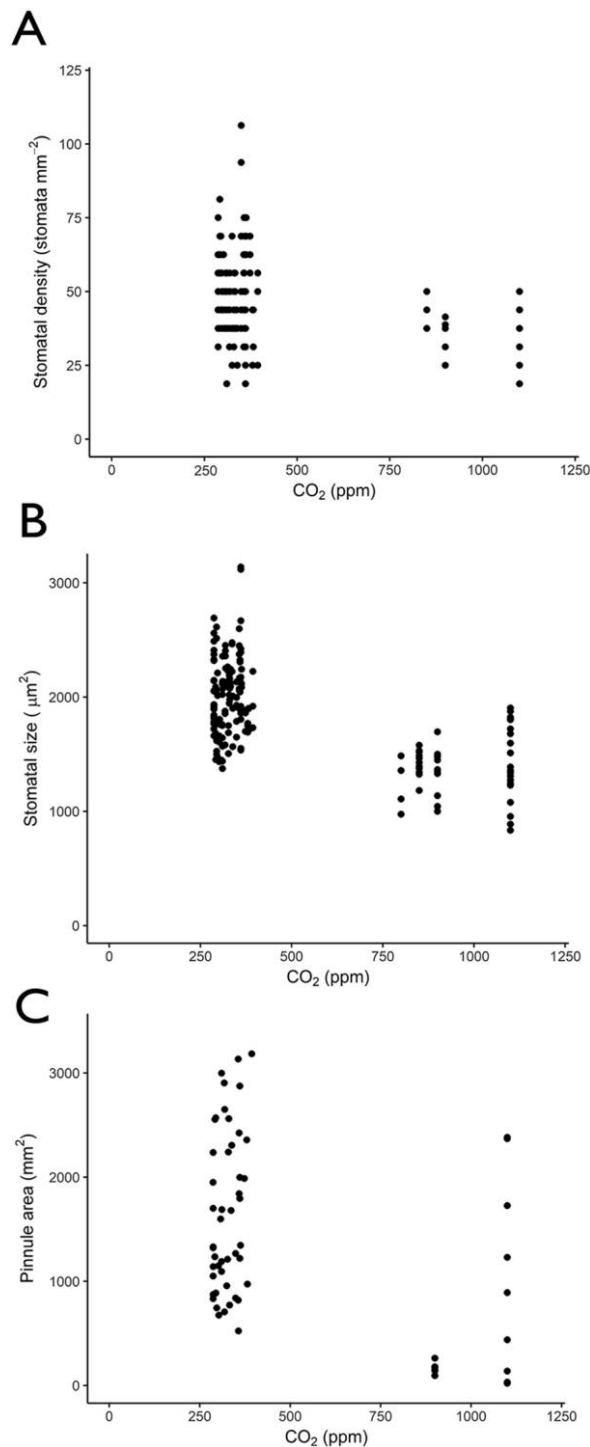


Fig. 9 Single-factor linear correlations between CO₂ and stomatal density (A; $R^2 = 0.4$, $P < 0.0001$), stomatal size (B; $R^2 = 0.11$, $P < 0.0001$), and pinnule area (C; $R^2 = 0.17$, $P < 0.0001$). Data points are individual measures.

to ash fall and acid precipitation in an environment with dry periods, although water bodies were present (Limarino et al. 2012). Hence, the recovery of *Bowenia* and *Eobowenia* fossils with relatively small pinnules was not expected. While only one

fragment of a pinnule has been recovered from Nerriga, there are several complete or near complete pinnules from Anglesea, and they are consistently small (Hill et al. 2018; fig. 2).

By contrast, the pinnules of *B. johnsonii* from Lowana Road in Tasmania are larger and not significantly different from the extant species (fig. 8). The Lowana Road vegetation was distinct from that at Anglesea and Nerriga, with evidence for mangrove vegetation (Pole and Macphail 1996), diverse broad-leaved angiosperms (Carpenter et al. 2007; Carpenter et al. 2012), diverse and common conifers (Bigwood and Hill 1985; Hill and Bigwood 1987; Hill 1990), and other gymnosperms (McLoughlin et al. 2008). The vegetation may have been open canopied (Pole 1998). It is possible that the size differences in the fossil *Bowenia* pinnules reflect the local conditions existing at the different sites.

At Nerriga and Anglesea, *Bowenia* were probably understory plants and needed to maximize sunfleck capture. While at the Anfiteatro de Ticó Formation, *E. incrassata* was shaded by ash fall; thus, it also needed to maximize sunfleck capture and minimize self-shading. In contrast, the more complex and probably open vegetation at Lowana Road may have driven the evolution of larger pinnules for the resident *Bowenia* species.

The high atmospheric CO₂ levels during the Eocene means that the Australian fossil plants were not CO₂ limited and could have a relatively low density of stomata. The thin boundary layer associated with the small pinnules would have allowed rapid dissipation of water vapor (Nobel 2009). This is potentially advantageous to the plant, as high humidity allowed the germination of fungal spores on the pinnule surfaces (see fig. 1F, 1G); thus, any air drying around the pinnule may have reduced fungal growth. The thin boundary layer of the Anfiteatro de Ticó Formation fossil species may have been an advantage during the warm conditions, as it increases convective heat dissipation (Martin et al. 1999).

Carbon dioxide concentrations during the Aptian were up to 2500 ppm (Wang et al. 2014), although they were more likely 1100 ppm and up to 1125 ppm during the Eocene (Lowenstein and Demicco 2006). Stomata were relatively small in the South American *E. incrassata* growing during the Aptian, and the Australian *Bowenia* pinnules growing during the Eocene had fewer and smaller stomata than the living species (fig. 8). Fewer diffusion sites means that there were fewer diffusion shells around stomata on each pinnule, and these were of a small size due to the small stomatal size (Nobel 2009). Physiologically, there are two likely explanations for this stomatal anatomy; one being that relatively small and few stomata can stay open all the time to allow rapid response to sunflecks, the other being that small stomata can open and close quickly to allow fast response to sunflecks for photosynthesis.

Along with pinnule area, stomatal anatomy may have assisted prevention of fungal growth. Few, small stomata assists avoidance of hyphal invasion through few, small invasion sites. Stomatal ratio, or the ratio of upper to lower stomata on an amphistomatic leaf, is driven by the trade-off between photosynthesis and pathogen resistance (Muir 2015). Although in this article there is not quantified stomatal ratio information, stomata are very rare on the adaxial surface. This trade-off suggests that small stomatal size decreases the likelihood that fungi will encounter a stomatal opening as a potential infection point. There is little information available on the life span of *Bowenia*

fronds, but prevention of fungal growth becomes more important the longer the fronds remain functional.

The presence of fewer, smaller stomata in the fossil species is a physical property that creates a short diffusion path length for CO₂ to enter and water vapor to escape, and the number of sites available for diffusion is reduced (Nobel 2009). Additionally, in both *E. incrassata* and fossil *Bowenia*, the small stomata may have been able to open quickly for sunfleck utility (Drake et al. 2013). Alternatively, rather than affecting CO₂ uptake or water loss, as both of these variables were abundant, stomata may have stayed open to allow photosynthesis as soon as a sunfleck hit the pinnule (Elliott-Kingston et al. 2016). The low irradiance at most of the fossil locations could mean that light was more limiting to photosynthesis than was CO₂.

We suggest that many climate and environmental factors have contributed to the stomatal and pinnule area changes of these species and that there is not one influencer. Thus, we are not using these data to create a proxy for paleo-CO₂ reconstructions. Carbon dioxide would have interacted with many other factors, and thus we deem a reconstruction inappropriate for these species.

This research supports published conclusions that plants at the Anfiteatro de Ticó Formation had limited light availability

due to ash fall during the Cretaceous, although the environment was open and water bodies were present. During the Eocene, the plant growth environment of southern Australia consisted of high humidity, low radiation, and high maximum temperatures. The morphology and anatomy of the pinnules allowed *Bowenia* and *Eobowenia* to survive in their environments, sometimes through small pinnules reducing self-shading and small and widely spaced stomata reducing the likelihood of fungal infection while still being available to open when light was sporadically available.

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