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A comparison of stomatal traits between contemporary and fossil leaves of Melaleuca quinquenervia: Do they reflect climate variation?

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Stomatal traits have been shown to vary in predictable ways in response to environmental change in many species. As a consequence, stomatal traits in fossil leaves are sometimes used as proxies for past CO2 and climate. Here we investigate the influence of temperature, rainfall and CO2 on stomatal traits in Melaleuca quinquenervia. We use both modern and sub-fossil leaves to evaluate the effect of CO2, and modern leaves for climate variables. We found a significant negative relationship between stomatal size and density across both modern and sub-fossil leaves of M. quinquenervia. However, we were unable to find any relationship between stomatal traits and CO2 across a range from 260-380 ppm. Using the modern data set we were unable to find any robust relationships between stomatal traits and either evaporation or temperature. Apogeotropic roots account for the lack of stomatal anatomy correlation to evaporation in a region that experiences inundation. We conclude that stomatal size is a highly plastic trait in this species and changes do not necessarily reflect functional changes in the leaves.

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Data for: A comparison of stomatal traits between contemporary and sub-fossil leaves of Melaleuca quinquenervia: do they reflect climate variation?

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Highlights

- Modern and subfossil leaves of the same species are analysed for stomatal variation
- Stomatal measures were higher and lower than the modern range
- No effect of climate on modern stomatal traits
- Stomatal size is a plastic trait of *Melaleuca quinquenervia*

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A comparison of stomatal traits between contemporary and sub-fossil leaves of *Melaleuca quinquenervia*: do they reflect climate variation?

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Abstract

Stomatal traits have been shown to vary in predictable ways in response to environmental change in many species. As a consequence, stomatal traits in fossil leaves are sometimes used as proxies for past CO_2 and climate. Here we investigate the influence of temperature, rainfall and CO_2 on stomatal traits in *Melaleuca quinquenervia*. We use both modern and sub-fossil leaves to evaluate the effect of CO_2, and modern leaves for climate variables. We found a significant negative relationship between stomatal size and density across both modern and sub-fossil leaves of *M. quinquenervia*. However, we were unable to find any relationship between stomatal traits and CO_2 across a range from 260-380 ppm. Using the modern data set we were unable to find any robust relationships between stomatal traits and either evaporation or temperature. Apogeotropic roots account for the lack of stomatal anatomy correlation to evaporation in a region that experiences inundation. We conclude that stomatal size is a highly plastic trait in this species and changes do not necessarily reflect functional changes in the leaves.

Key words: Stomatal density, stomatal size, climate proxy, Holocene, *Melaleuca quinquenervia*.

1. Introduction

Plants are capable of morphological and physiological plasticity in response to a range of conditions. Plasticity maximises survival in the face of environmental variables such as light, rainfall, humidity, temperature, CO_2 and nutrients. Plant plasticity manifests in a number of ways including changes in leaf size and shape, and stomatal traits that can often be measured in fossils. Stomatal changes influence the maximum potential uptake of CO_2 and control the rate of water loss in leaves. Maximum potential water loss through stomata (g_{wmax}) is a function of stomatal size and density, and is directly related to stomatal conductance for a range of plants. For example, Feild et al. (2011) analysed 87 basal angiosperm species from across the world and found a strong, positive relationship between g_{wmax} and measured stomatal conductance. Maximum potential water loss through stomata determines the upper limit to stomatal conductance and therefore limits photosynthesis and whole canopy gas exchange (de Boer et al., 2011). It can vary in response to changing environmental conditions; for example, it has been shown to increase with decreasing CO_2 thereby increasing the capacity for CO_2 uptake as availability declines (Franks et al., 2012). The sensitivity of g_{wmax} to changing atmospheric CO_2 concentration across the last 400 My has been demonstrated for almost all plant groups, from non-vascular plants to angiosperms (Franks and Beerling, 2009). This g_{wmax} response has also been experimentally demonstrated in living specimens of *Commelina communis*, *Vicia faba*, *Osmunda regalis* and *Selaginella uncinata* grown in growth chambers with CO_2 concentrations of 760 to 820 ppm (Franks et al., 2012).

Stomatal size and density are affected by environmental factors other than CO_2. These include water availability (Fraser et al., 2009), temperature (Beerling and Chaloner, 1993), nutrients (Peñuelas and Matamala, 1990), light (Onwueme and Johnston, 2000), soil salinity (Bray and Reid, 2002) and humidity (Nejad and Van Meeteren, 2005). Thus, stomatal traits are often used as proxies to infer past climate.

There have been times in the past when the atmospheric CO_2 concentration has been relatively stable. One of these periods was the Holocene epoch when CO_2 changed by less than 20 ppm over the 11 700 years prior to ~1850 AD (Indermühle et al., 1999). Therefore, changes in stomatal traits over this time may be due to other environmental factors, such as temperature or rainfall variation. Considerable global and regional scale climatic variability occurred through the Holocene (Wanner et al., 2011). For example, the northern Australasian monsoon was enhanced between 7500 and 5000 years before present (BP), resulting in high rainfall, at times above that of the modern range (Shulmeister and Lees, 1995). In a review of eastern Australian mid-Holocene rainfall variability, Reeves et al. (2013) found that the majority of studies indicate a decline in effective precipitation after 5000 BP. These proposed rainfall fluctuations may have influenced stomatal traits across the Holocene.

The relative stability of atmospheric CO_2 during the Holocene provides an opportunity to examine how stomatal traits may have varied over this time in response to temperature and class A pan evaporation (hereafter, evaporation). In this study, we investigated responses of *Melaleuca quinquenervia* (Cav.) ST Blake to a range of environmental variables. Leaves of *M. quinquenervia* from core samples obtained from both lake sediments (hereafter referred to as sub-fossil) and modern leaf litter (hereafter referred to as the modern dataset) are tested. This is the first study that we know of that incorporates both a long-term leaf litter collection and sub-fossil specimens of the same species and analyses their response to environmental variables.

2. Methods

2.1 Study site and species

Melaleuca quinquenervia is an Australian native tree species that occurs on flood plains, wetlands (Greenway, 1994), and littoral zones of lakes (Lockhart et al., 1999). When inundated, the tree grows roots from epicormic buds in an upward direction thus allowing oxygen uptake into roots (McJannet, 2008). It is categorised as scleromorphic, based on features such as fibrous leaves with a thick cuticle, and an evergreen habit (Hill, 1998). *M. quinquenervia* occurs along the east coast of Australia in Queensland and New South Wales (Brophy et al., 2013). It also occurs naturally in Indonesia, Papua New Guinea, and New Caledonia (Brophy et al., 2013), the Hawaiian Islands and has naturalised in southern Florida where it is considered an invasive species (Pratt et al., 2014).

We measured stomatal traits from *M. quinquenervia* leaves collected between 1992 and 2003 from Carbrook Wetland (27.7°S, 153.2°E), a seasonally inundated wetland approximately 40 km south of Brisbane (Fig. 1; Greenway, 1994). We also measured stomatal traits of sub-fossil leaves obtained from a sediment core taken from Swallow Lagoon on North Stradbroke Island, Queensland, Australia (27.5°S, 153.4°E). Swallow Lagoon is a small (0.27 ha), oligotrophic, freshwater lake located high (94 m above sea level) in the dunes of North Stradbroke Island, the World's second largest sand island. The lake is perched, meaning that it is separated from the regional water table. Variation in water depth is therefore a function of the balance between precipitation and evaporation. Extant populations of *M. quinquenervia* continue to grow as fringing vegetation around the lake.

As the modern specimens all came from Carbrook Wetland and all sub-fossil specimens came from Swallow Lagoon, we assume that nutrient availability and soil salinity were similar for all modern and all sub-fossil leaves collected for this study. We also assume that light availability did not affect leaves as the plants we studied have open canopies and do not have significant self-shading.

2.2 Modern leaves

Modern *M. quinquenervia* leaves were taken from samples collected for a leaf-litter monitoring project, the initial results of which are reported in Greenway (1994). Leaf litter was collected in a litter trap at four-week intervals, between April, 1992 and July, 2003, from Carbrook Wetland (Fig. 1). For our study, one leaf was sampled every second month, beginning in April 1992 with an even spread of sampling conducted over the twelve year time frame (n = 4, 5 or 6 per year). A total of 61 leaves were analysed.

2.3 Sub-fossil samples

Sediment cores were taken from a platform, anchored over the deepest part of Swallow Lagoon, in March, 2011. The record is a composite of two cores. Core SL1 (150 - 375 cm), which was collected using a Livingstone corer, was extruded in the field where it was sealed and stored for transportation back to the laboratory for analysis. Core SLP3 (0 - 250 cm) was collected using a clear perspex soft sediment piston corer. This was extruded in the field at 1 cm increments from 0 to 150 cm, and the remainder as a single section. The two cores were correlated stratigraphically, using a distinct 5 cm thick band of sand evident in both cores at a depth of 220 cm.

The cores were sampled at contiguous 1 cm intervals and the sediment was washed through a 500 µm sieve to obtain macrofossil remains. The retained fraction was rinsed in distilled water and *M. quinquenervia* leaf fragments were selected for analysis. Fragments of *M. quinquenervia* were identified using distinctive morphological features, such as thick veins, epidermal colour and anatomy of the spongy mesophyll. A chronology for the core, based on twelve ^{14}C dates on terrestrial leaf macrofossils, indicates the sediment covers the last 7300 years (Tibby et al., 2016). The distribution of leaves varied through the sediment column and specimens were collected where samples of sufficient size and number were available. A total of 93 leaf fragments were analysed.

2.4 Cuticle preparation

2.4.1 Modern leaves

One cm^2 pieces were cut from the leaf margin half way along the lamina. These were placed in separate test tubes, covered in 80% ethanol v/v, and left for 24 hours at room temperature. The ethanol was then removed and leaves covered in a 2:1 solution of 35% hydrogen peroxide and 80% ethanol (v/v). The test tubes were then placed in a water bath in a fume cupboard and gently heated until the leaf samples were translucent, indicating that the cuticle had separated from the rest of the leaf. The leaf samples were gently rinsed with reverse osmosis (RO) water, and debris was brushed away from the cuticle with a fine camel hair brush. The cuticles were stained with 0.05% crystal violet (w/v) for 10 seconds. Leaf cuticles were transferred to a slide, and mounted in warm phenol glycerine jelly. A coverslip was placed on top of the sample and left overnight at room temperature. Nail polish was then applied to the coverslip edges to preserve the cuticles from dehydration.

2.4.2 Sub-fossil samples

Leaf fragments were covered in a solution of 10% aqueous chromium trioxide (w/v), and left for between 24 hours and 5 days at room temperature. The resulting leaf cuticles were rinsed in RO water, then stained and then mounted onto slides using the same method as described for the modern leaves. Fossil cuticle slides are stored at The University of Adelaide collection.

2.5 Stomatal measurements

Leaf cuticles were examined with a light microscope (Olympus AX70), and photomicrographs were taken with an Olympus UC50 camera. Micrographs were analysed using imaging software (AnalySIS version 6.0.6001 Service Pack 1 Build 6001, Acer, Australia). In the case of the sub-fossil leaves, three pieces of leaf cuticle per one cm of leaf core section were measured; these pieces of leaf cuticle were often fragmentary.

Stomatal size (μm^2) was calculated as the mean of length by width of five guard cell pairs per piece of cuticle. Stomatal density (stomata mm^{-2}) was determined by counting the number of stomata in a minimum 100 x 100 μm area on each cuticle. Where possible, a 200 x 200 μm area was used for stomatal counts with three of these areas counted per cuticle. Three of the one cm core sections had only one leaf fragment available for counting density, and three had only two leaf fragments available for this purpose. Final numbers for stomatal density were n=62, and for stomatal size were n=93. Stomatal density had fewer measurements as some leaf pieces were too small to determine stomatal density but we were able to measure stomatal size.

For the modern leaves we present individual measurements of the abaxial and adaxial leaf surfaces for stomatal density and size and compare these with stomatal traits from sub-fossil samples.

2.6 Environmental data

Environmental data were obtained using the Scientific Information for Land Owners (SILO) database for the location 27.7°S, 153.2°E. SILO is a database compiled and interpolated from observational data collected by the Australian Bureau of Meteorology (Jeffrey et al., 2001) The data obtained from SILO were mean annual values for minimum air temperature (°C), and class A pan evaporation (mm year^{-1}).

The mean annual minimum temperature ranged from 14.1°C for the 1994 dataset to 15.7°C for the 1998 dataset. Class A pan evaporation ranged from 3.97 mm year^{-1} in 1999 to 4.66 mm year^{-1} in 1994. As leaf longevity for *M. quinquenervia* is 2-4 years (M. Greenway, Griffith University, Australia pers. comm; Van et al., 2002), environmental data from two years prior to leaf litter collection have been compared with stomatal data.

2.7 Statistical analyses

All statistical analyses have been run in RStudio. Locally Weighted Least Squares Regression (loess) shows smoothed relationships between stomatal density and size for both modern and sub-fossil specimens. Loess also shows smoothed relationships between stomatal size and climate variables.

ANOVAs have been performed to test significance between the measures of sub-fossil and modern populations of stomatal size and density.

3. Results

3.1 Stomatal size and density

The difference between sub-fossil and modern stomatal density, size is not statistically significant. Modern and sub-fossil differences are as follows: stomatal density $P = 0.264$; stomatal size $P = 0.327$. Notably, there was a greater range in values for stomatal size and density in the sub-fossil data set than the modern one (Figs. 5 and 6). The sub-fossil leaves contained the largest and smallest stomata, and likewise the highest and lowest stomatal densities. For the combined modern and sub-fossil data sets, there was a negative relationship between stomatal size and density (Fig. 3).

3.2 Correlation of stomatal size to climate

The assumptions for linearity were not satisfied for any relationships discussed here, thus any correlations can only be considered as general trends. Stomatal size of leaves from the modern dataset was weakly, but significantly correlated with class A pan evaporation (Fig. 4). Variation in class A pan evaporation explained 8.1% of the variation in stomatal size for *M. quinquenervia*. Stomatal density did not correlate with class A pan evaporation. There were also no correlations between stomatal size or density from the modern dataset with mean daily minimum temperature. There is a weak correlation between minimum temperature and stomatal size; minimum temperature explained 5.6% of the variation in stomatal size.

4. Discussion

This study investigated relationships between stomatal morphology and temperature, evaporation and CO_2 using *M. quinquenervia* leaf litter collected over twelve years from 1992 to 2003. We also measured stomatal traits of sub-fossils of the same species collected from a lake covering the period from 7300 years ago to 1975 AD.

4.1 Stomatal density and size

The negative relationship we observed between stomatal density and size of both the modern and sub-fossil *Melaleuca quinquenervia* leaves was similar to observations reported in previous studies on different species. These studies have shown a negative, logarithmic relationship between stomatal size and density (for example Brodribb et al., 2013; Franks and Beerling, 2009). This relationship between stomatal size and density is likely to be due to the limited leaf area that is available for stomata (Brodribb et al., 2013), but it also has functional significance as more, smaller stomata result in a higher maximum potential water loss than fewer, larger stomata with deeper pores (Brodribb et al., 2013; Franks and Beerling, 2009).

4.2 Climate correlations

Minimum temperature was weakly correlated with short-term changes in stomatal size in the modern *M. quinquenervia* leaves (Fig. 3). A larger stoma creates a longer diffusion path for water to travel along when exiting the leaf during transpiration (Nobel, 2009), and thus reduces transpiration rates. Our analyses showed that larger stomata in *M. quinquenervia* leaves formed during periods of warmer minimum temperatures. Temperature is directly proportional to vapour pressure deficit, and warmer minimum temperatures create a larger VPD and thus a greater driving force for water to evaporate through open stomata. For a given VPD, larger stomata would slow evaporation relative to smaller stomata, and thus retain water for longer.

There was no correlation between stomatal density and temperature, thus, it could be argued that there was no functional change in potential water loss as only stomatal size changed weakly in response to temperature. The lack of response by stomatal density to temperature leads to the hypothesis that stomatal size is a more plastic phenotype than the former two variables with temperature changes.

There is no correlation between stomatal size and evaporation. It is possible, however, that stomatal size in leaves of *M. quinquenervia* is unrelated to evaporation because it frequently occurs in areas where the water table is high (e.g. wetlands and lake edges). It also has apogeotropic roots that are adapted to rising and falling water tables. McJannet (2008) demonstrated that stand transpiration of *M. quinquenervia* was unaffected by variation in water table depth due to these root systems. Thus, in this high water environment, evaporation is unlikely to be a limiting factor.

Throughout the 7300 year Holocene leaf accumulation, the stomatal size of the sub-fossil samples regularly fall outside the range of that of the modern dataset. The correlation of stomatal size of the modern dataset to minimum temperature likely reflects the correlation between these variables during the Holocene, although it is important to note the temporal range between these two is quite different, since we are comparing a 12 year data set with a 7500 year one and the range of temperatures and evaporation will be different, but we do not know the extent of this. As such, the weak correlation between stomatal size and minimum temperature can not be considered for use as a palaeo-temperature proxy. Change in stomatal anatomy has been shown to correlate with CO_2 changes though we do not consider this to be the case for change in *M. quinquenervia* stomatal size as CO_2 only increased by ~20ppm during the Holocene (Indermühle et al., 1999).

During the Holocene, large changes in El Niño-Southern Oscillation (ENSO) caused variability in rainfall events; an intensification of ENSO in the late-Holocene has been noted in a range of proxies from the Eastern Pacific (for example Conroy et al., 2008; Koutavas and Joanides, 2012; Moy et al., 2002) and Australia (for example Barr et al., 2019; Donders et al., 2007; Quigley et al., 2010; Shulmeister and Lees, 1995). However, an intensification of ENSO leading to larger rainfall events mediated by *M. quinquenervia*'s root system is thus not reflected in stomatal size during the Holocene. There is evidence that there were cold periods in other parts of the world apart from Stradbroke Island during the Holocene (Wanner et al., 2011). Barr et al. (2019) used sediments from the same species in the same region of Australia to calculate a rainfall proxy for Stradbroke Island during the Holocene. These authors were able to use carbon isotope values to do this reconstruction and our data are not similar to theirs (Barr et al., 2019) as we were unable to demonstrate a rainfall response in stomatal density or size and thus could not reconstruct a proxy for rainfall. Potential reasons for this include stomatal size and density responses occuring at a longer time scale-years- than carbon isotopes-weeks. It is also possible that *M. quinquenervia* stomata are not that sensitive to environmental changes, whereas carbon isotope composition is sensitive to rainfall variation. Finally, it is always going to be more difficult to obtain stomatal data because of the limited amount of sub-fossil material available, whereas the *M quinquenervia* C isotope data were obtained from sediment cores. Thus, is that carbon isotope composition may be a better proxy than stomata.

However, more evidence of temperature and evaporation forcing on stomatal morphology is required before we can use these data to create proxies of Holocene climate. The relationships found here are weak for temperature and evaporation response and more data are needed to detect subtle correlations with climate. These include collection of herbarium data from a wider spatial and temporal range, including invasive *M. quinquenervia* from Florida and an increase in the sample size of sub-fossil leaves.

4.3 Conclusion

This study showed that stomatal size correlated with minimum temperatures indicating a response by leaf anatomy to changes in VPD. Apogeotropic roots account for the lack of stomatal anatomy correlation to evaporation in a region that experiences inundation. The sub-fossil dataset indicates that there may have been climate influences forcing stomatal change, however, we did not find stomatal size or density to be reliable proxies of palaeo-environments. Analysis of a larger dataset of sub-fossil and modern leaves is required to detect any more subtle correlations between stomatal anatomy and climate variables.

5. Acknowledgements

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7. List of Figures and Tables

Table 1. Ranges and means of stomatal parameters for modern and sub-fossil datasets of *Melaleuca quinquenervia*. For the modern dataset, both stomatal size and density have the same number of observations, n= 126. For the sub-fossil material stomatal density measurements, n = 177, for the sub-fossil material stomatal size measurements n = 184.

Figure 1. Location of field sites where the modern leaves (Carbrook) and sub-fossil leaves (Swallow Lagoon) of *M. quinquenervia* were collected (map modified from Figure 1c of Tibby et al., 2016).

Figure 2. The relationship between stomatal density and size for modern (closed circles) and sub-fossil (open circles) leaves of *M. quinquenervia.*The line is a loess smoothed model.

Figure 3. Relationship between mean daily minimum temperature and stomatal size is a smoothed loess line for the modern *M. quinquenervia* leaves (Multiple R-squared = 0.08, P = 0.001).

Figure 4. Relationship between class A pan evaporation and stomatal size is a smoothed loess line for the modern *M. quinquenervia* leaves (Multiple R-squared = 0.056, P = 0.007).

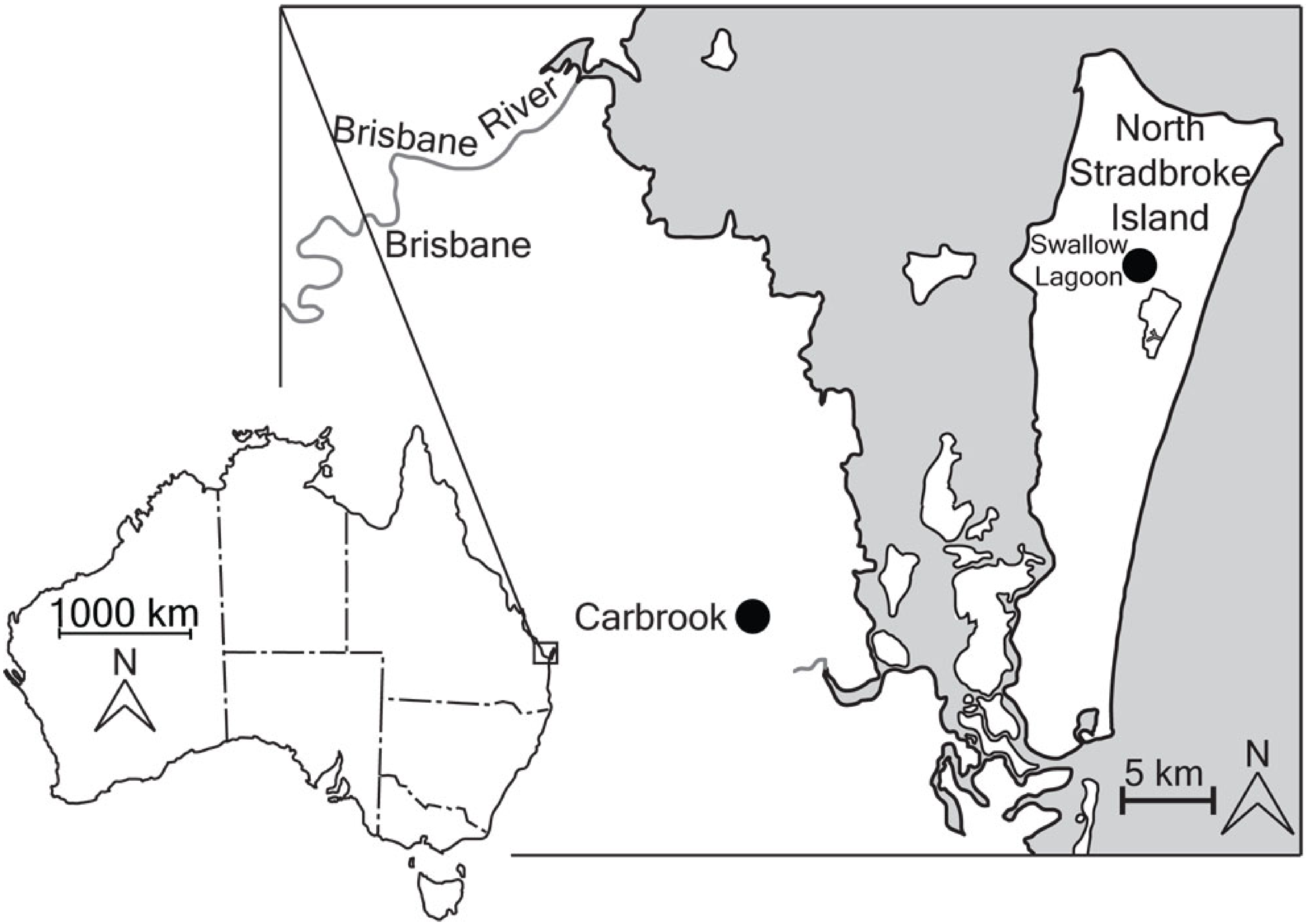
Figure 5. Changes in stomatal density during the Holocene for *M. quinquenervia* from sub-fossil samples. The dotted lines indicate the maximum and minimum values of stomatal density, for the modern dataset (Table 1).

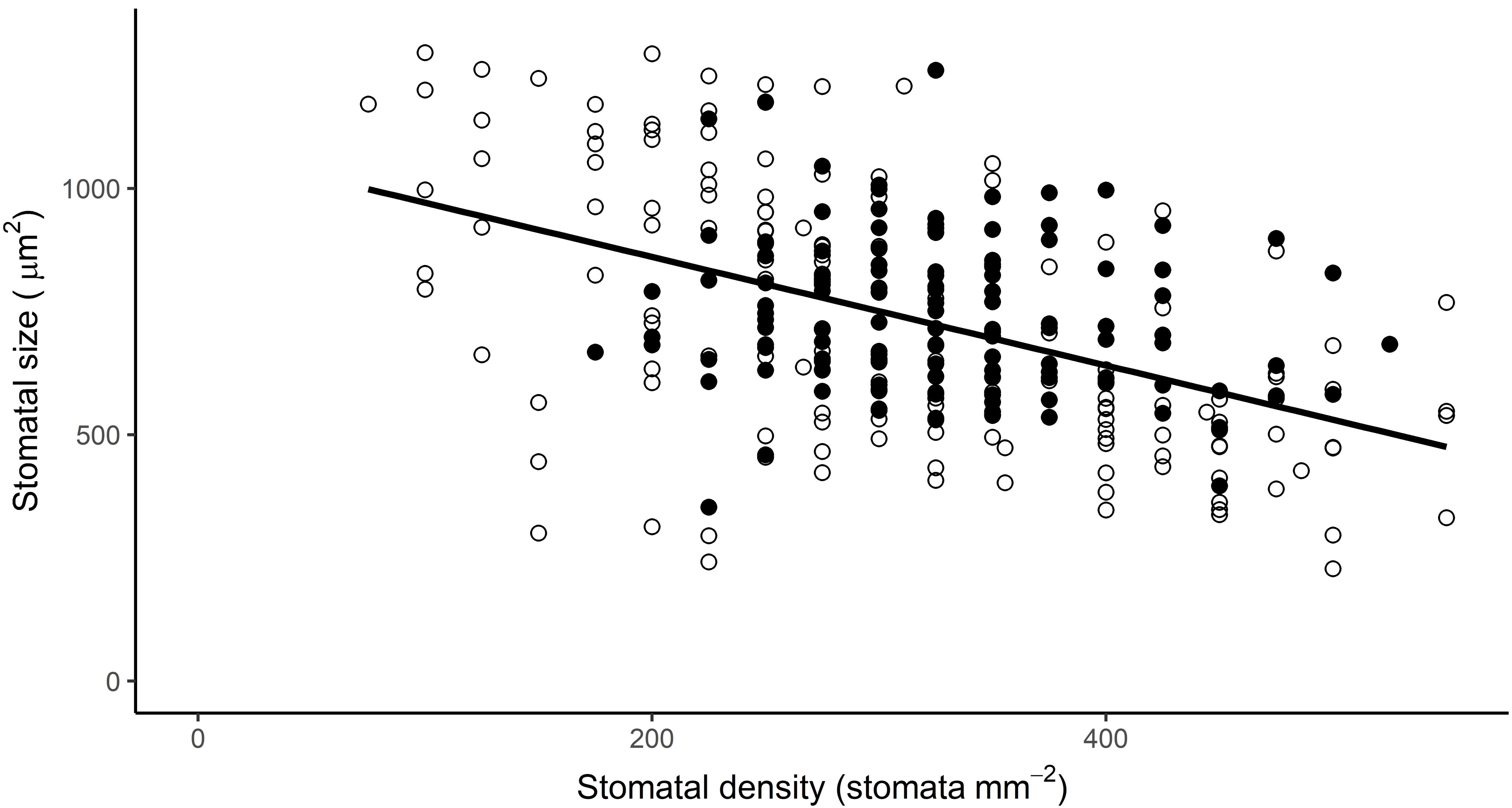
Figure 6. Changes in stomatal size during the Holocene for *M. quinquenervia* from sub-fossil samples. The dotted lines indicate the maximum and minimum values of stomatal size, for the modern dataset (Table 1).

8. Tables

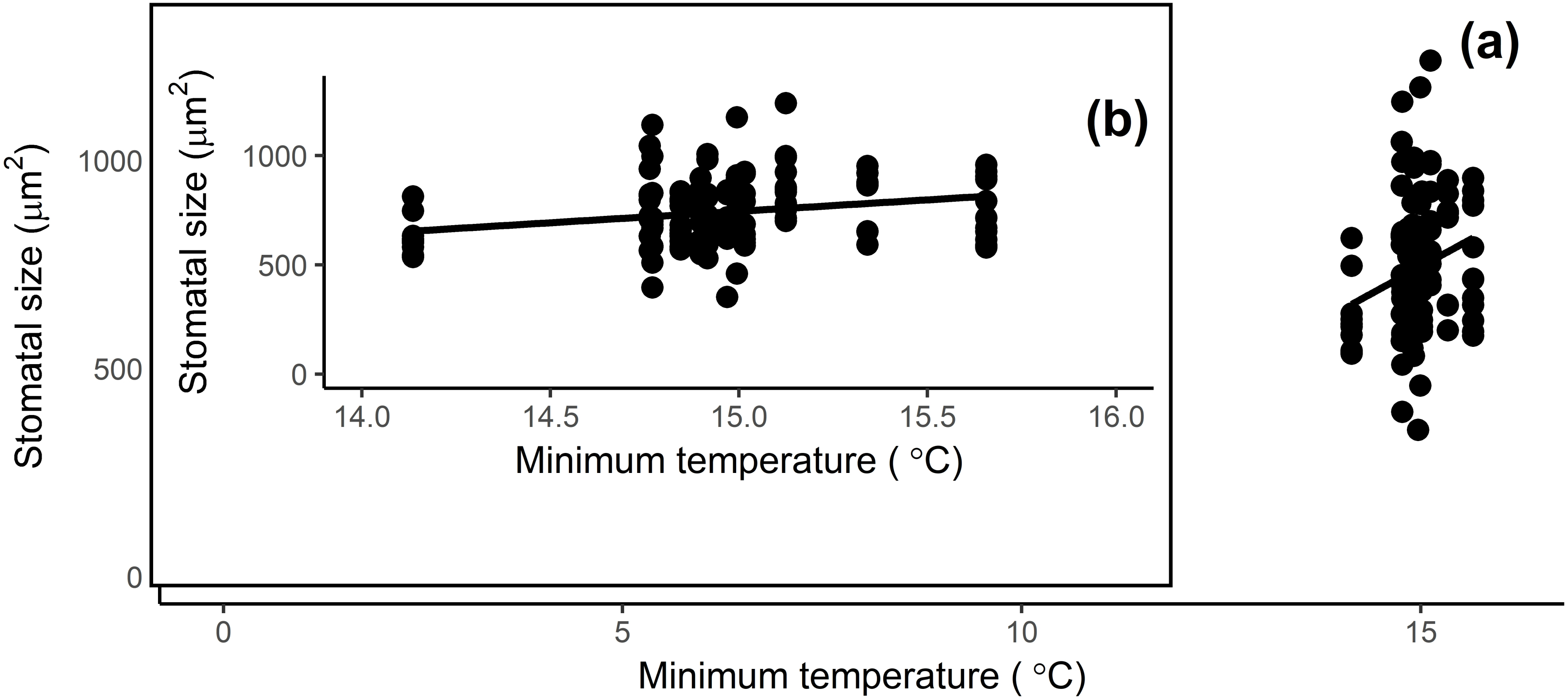
Table 1.

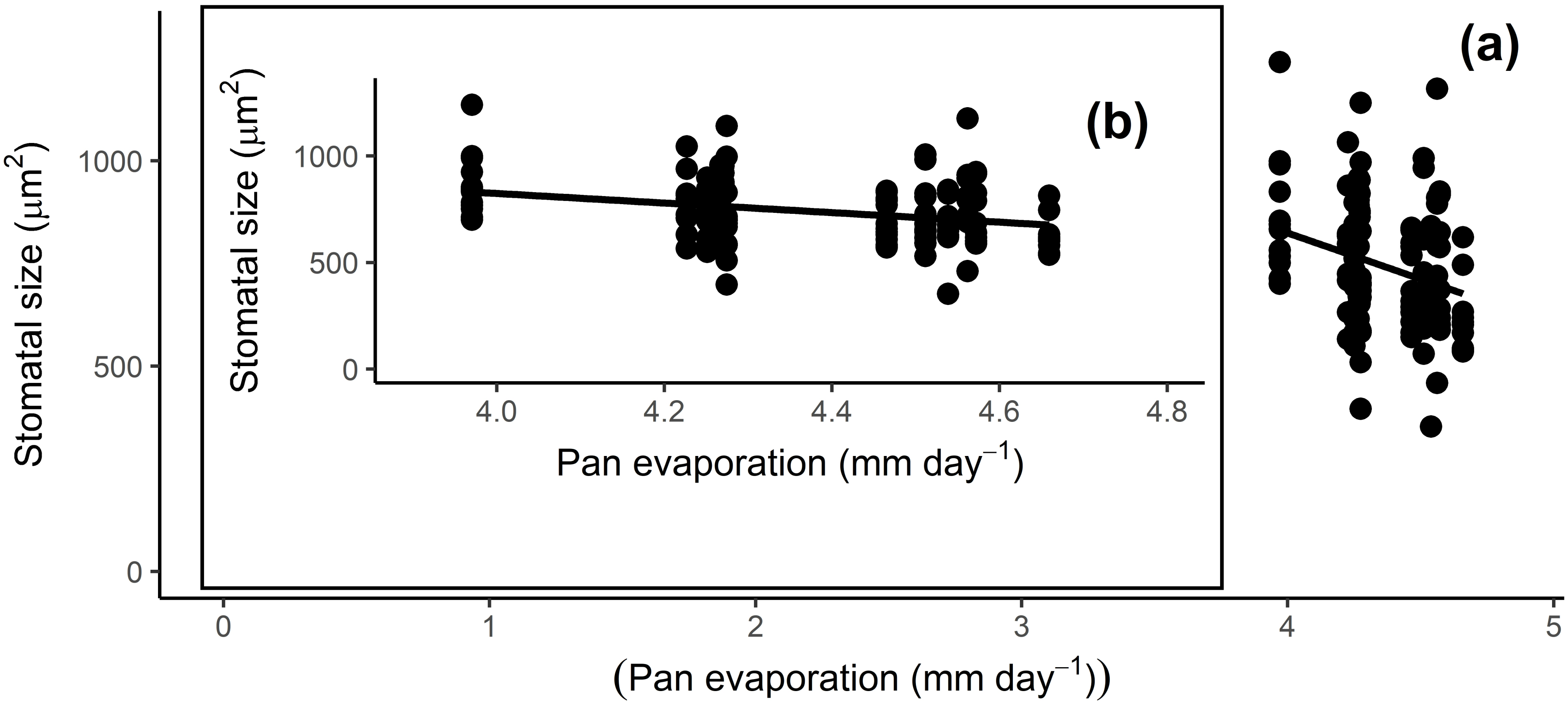
	Modern data set			Sub-fossil data set		
	Min.	Max.	Mean ± SE	Min.	Max.	Mean ± SE
Stomatal density (stomata mm^{-2})	175	525	327 ± 6	75	900	344 ± 12
Stomatal size (μm^2)	353	1240	740 ± 13	139	1699	710 ± 24

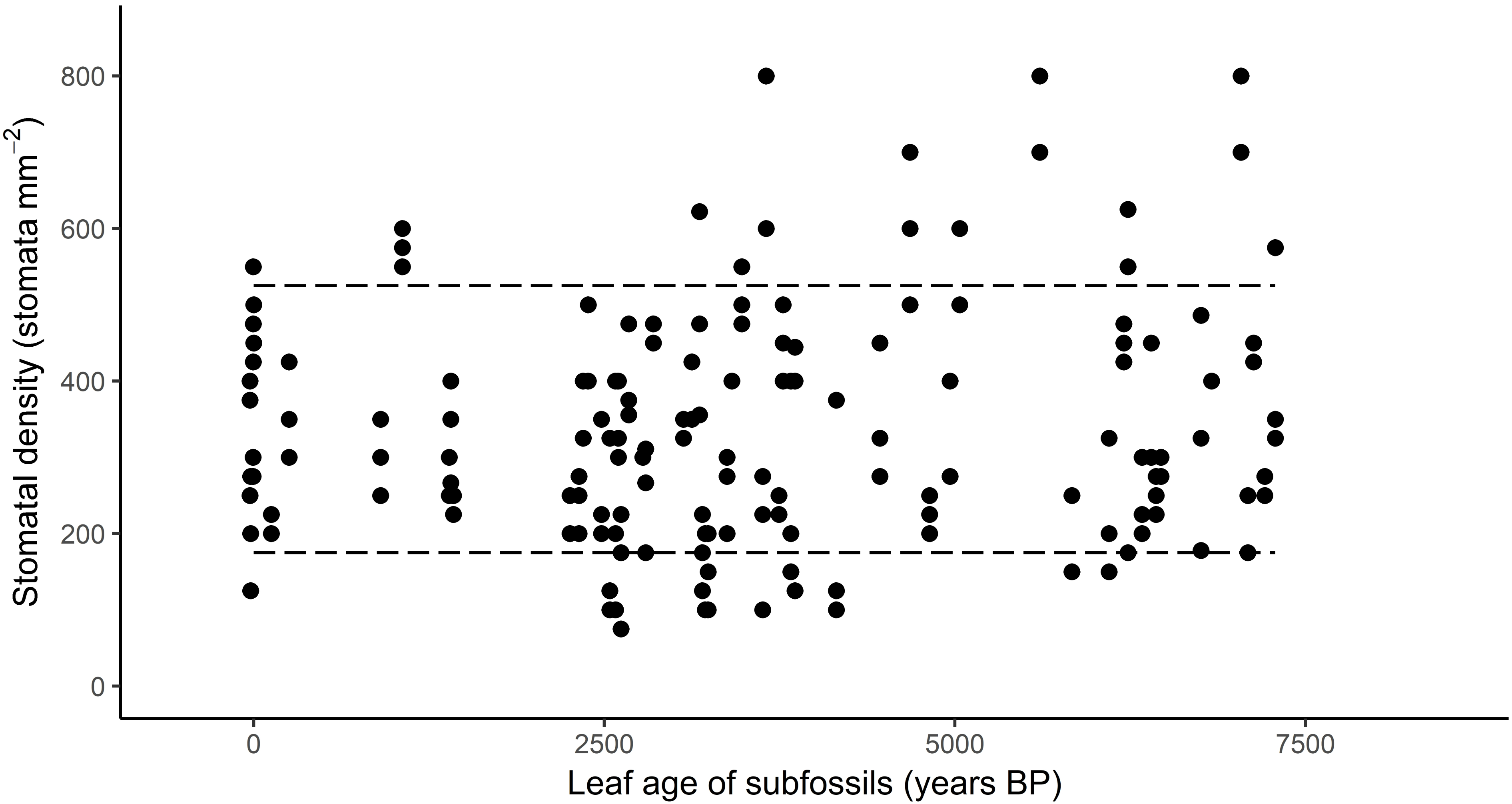

Brisbane River
Brisbane
North Stradbroke Island
Swallow Lagoon
Carbrook
1000 km
N
5 km
N



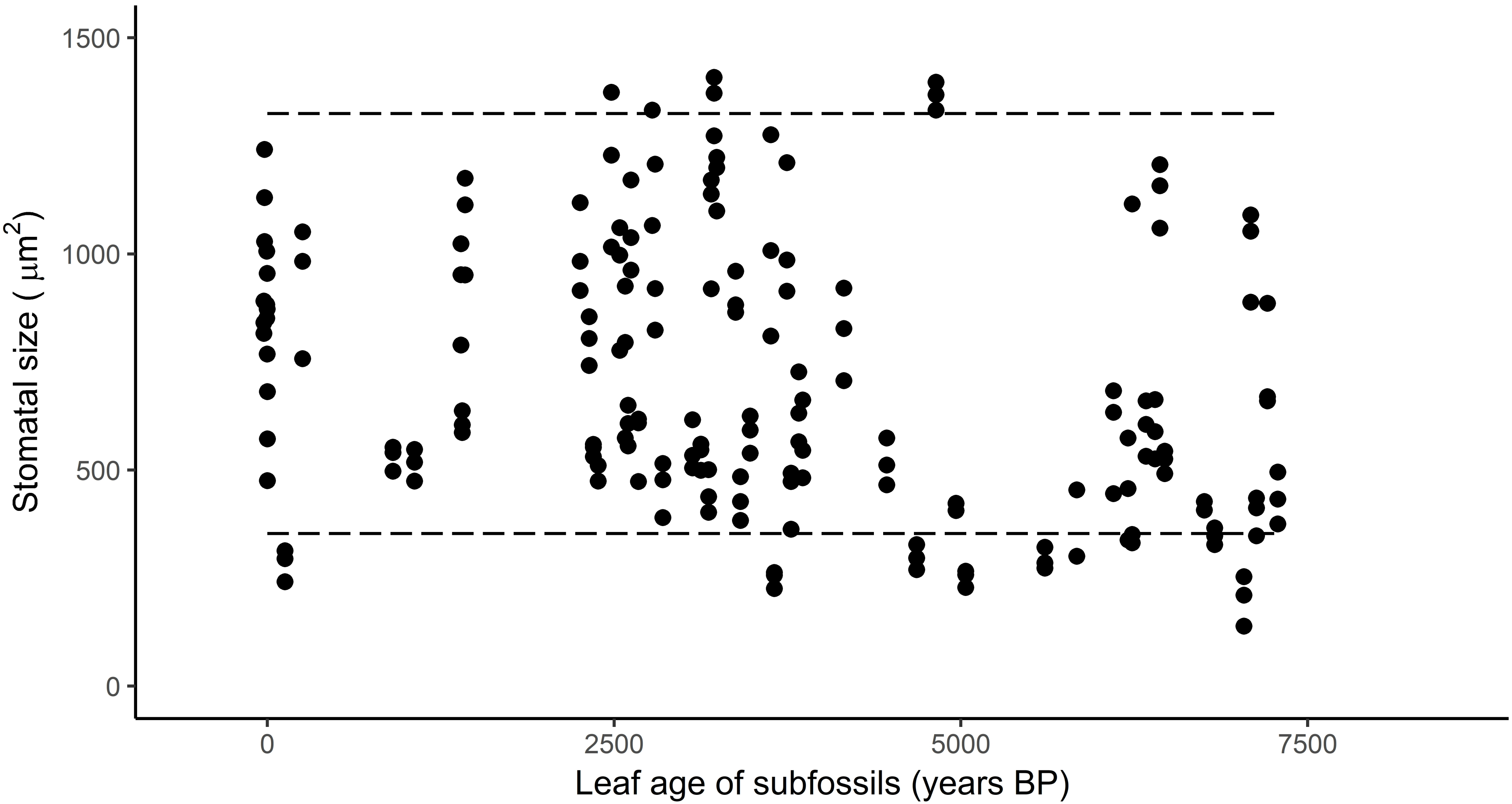
Stomatal size (μm2)
1000
500
0
0
200
400
Stomatal density (stomata mm−2)


(a)
(b)
Stomatal size (μm²)
1000
500
0
14.0
14.5
15.0
15.5
16.0
Minimum temperature (°C)
Stomatal size (μm²)
1000
500
0
0
5
10
15
Minimum temperature (°C)


(a)
(b)
Stomatal size (μm²)
1000
500
0
4.0
4.2
4.4
4.6
4.8
Pan evaporation (mm day⁻¹)
Stomatal size (μm²)
1000
500
0
0
1
2
3
4
5
(Pan evaporation (mm day⁻¹))



Stomatal density (stomata mm^{-2})
800
600
400
200
0
0
2500
5000
7500
Leaf age of subfossils (years BP)



Stomatal size (μm^2)
1500
1000
500
0
0
2500
5000
7500
Leaf age of subfossils (years BP)