Intrinsic variation associated with stomatal frequency within Victorian specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*

A preliminary study: implications for palaeoclimatological reconstructions.

Mark J. Scarr, David R. Greenwood, Carol Scarpaci

Mark J. Scarr (corresponding author): School of Engineering and Science, Victoria University, PO BOX 14428 (St Albans Campus) MCMC 8001, Victoria, Australia. Fax 61 3 9919 2465
Email:mark.scarr@vu.edu.au
David R. Greenwood: Department of Biology, Brandon University, 270 18th Street, Brandon, Manitoba, Canada, R7A 6A9
E-mail:greenwoodd@brandonu.ca
Fax: (204) 728-7346
Carol Scarpaci: School of Engineering and Science, Victoria University, PO BOX 14428 (St Albans Campus) MCMC 8001, Victoria, Australia. Fax 61 3 9919 2465
Email:carol.scarpaci@vu.edu.au

Abstract

Intrinsic variation in stomatal frequency, density and index, was assessed in 3 Australian tree species, *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to determine the applicability of these leaf characters to be applied as palaeoclimatological tools. Intrinsic variation was assessed among leaves, over the leaf surface, across the leaf surface and between leaf surfaces. Stomatal index in *A. melanoxylon* did not show any significant intrinsic variation in any of the test factors, stomatal density of *A. melanoxylon* varied significantly among leaves and over the leaf. Stomatal density and index in *A. smithii* only varied significantly among leaves, while *E. obliqua* exhibited significant variation in stomatal density and index among leaves, and between leaf surfaces. Stomatal index of *A. melanoxylon* is a potential candidate for the use in palaeoclimatological reconstructions.

Introduction

Stomates or stomata are pores upon the leaf surface that allow gas exchange between the leaf and atmosphere. It is the movement of carbon dioxide [CO_2] from the atmosphere into the leaf, via these pores, that permits the photosynthetic reaction to occur (Beerling and Chaloner, 1992; Royer, 2001). Stomatal frequency refers to the number of stomata distributed across the leaf surface and it may be defined as a density count, stomata density per mm^2, or expressed as an index which calculates the ratio of stomata to epidermal cells (Salisbury, 1927; Greenwood et al., 2003).

Leaf morphological characters, such as stomatal frequency, have been found to respond to changing environmental variables, such as, temperature, rainfall, irradiance and [CO_2] (Givnish, 1978; Greenwood, 1992; Royer, 2001). If stomatal frequency is able to respond to a particular environmental or climatic variable in a predictable manner, this character holds the potential to be applied as a palaeoclimatological tool; thus potentially providing past climate estimates (Woodcock, 1992; Wing and Greenwood, 1993). The applicability of such a relationship is dependent upon the selected characters ability to accurately track a changing environmental variable and the characters ability not to be obscured by other factors. Therefore, documenting a range of factors that can affect the efficiency of utilising a chosen characteristic (i.e. stomatal frequency) to document past climate change is crucial.

Intrinsic variation is a factor that may obscure the potential use of stomatal frequency as a palaeoclimatological tool (Körner, 1988; Wagner et al., 2005). In terms of stomatal frequency, intrinsic variation is the variability in stomatal distribution across a leaf surface (Poole and Kürschner, 1999), and has the potential to be large in angiosperms (Poole *et al.*, 1996 & 2000, Kouwenberg et al., 2004; Uhl and Kerp, 2005). Typically, variation in stomatal frequency has been found to vary from the base to tip, midrib to margin, between surfaces of a leaf, within a leaf fragment and between individual leaves; with stomatal density exhibiting greater intrinsic variability than stomatal index (Salisbury, 1927; Sharma and Dunn, 1968; James and Bell, 1995; Ferris et al., 1996; Smith et al., 1989; Zacchini et al., 1997; Stancato et al., 1999; Royer, 2001). So if stomatal frequency is to be used as a proxy-estimator of some past climate parameter, the degree of intrinsic variation needs to be determined, as will be undertaken in this paper.

Australia fossil leaves, including the use of stomatal frequency, have been employed to estimate palaeoclimatic parameters, such as, temperature, precipitation and [CO_2] (Greenwood and Wing, 1995; Atchison and Head, 1999; Atchison et al., 2000; Greenwood et al., 2003; Carpenter et al., 2004; Greenwood et al., 2004). Greenwood et al. (2003) is the only Australian study to use stomatal index from fossil specimens, *Litsea* a tropical rainforest tree, to estimate Eocene [CO_2]. The use of stomatal frequency analysis

Species	Latitudinal Range (°S)	Altitudinal Range (m)	Annual Temperature Range (°C)	Annual Precipitation Range (mm)
Acacia melanoxylon	14 – 43	0 – 1500	1 – 30	750 – 7500
Acmena smithii	11 – 39	0 – 1200	5 – 32	700 – 2000
Eucalyptus obliqua	28 - 43	0 – 1200	-4 – 29	500 – 2400

Table 1: Physical and climatic parameters associated with distributions of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*. Source: Boland *et al.*, 1984.

has the potential to provide valuable information on past [CO_2], this information may be used to provide an insight into how future atmospheric [CO_2] may affect the Australian landscape.

The species to be examined in this study include two mesic species, *A. melanoxylon (R. Br.)* and *E. obliqua (L'Hér.)*, and one warm temperate rainforest species, *A. smithii (Poiret) Merr. & Perry var. smithii.* These species were chosen based on their broad environmental range in Victoria (Table 1), which allows establishment of wide environmental transects, thus permitting the assessment of stomatal frequency response to changing environmental and climatic parameters. *Acacia melanoxylon* does have a fossil record (Jordan, 1997),

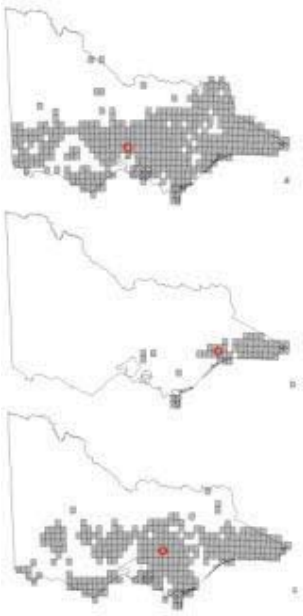

Figure 1: Victorian distribution and collection sites of a) *Acacia melanoxylon*, b) *Acmena smithii* and c) *Eucalyptus obliqua* (Source: Gullan, 1998).

while *A. smithii* and *E. obliqua* have not currently been identified in the fossil record, although *Eucalyptus* and *Acmena* specimens do exist, so there is potential for these species to be described in the future (Greenwood et al., 1997; Blackburn and Sluiter, 1994). Identification of fossil specimens to species level may be achieved by the use of, or combination of bark / wood, seeds, fruit, pollen, leaf architecture, cuticle morphology and DNA analysis (Hickey, 1979; Christophel and Rowett, 1996; Ladiges et al, 2003; Hopper and Gioia, 2004; Basinger et al, 2007). Currently, there is only one study examining the response of stomatal frequency in *A. melanoxylon* to increasing atmospheric [CO_2] (Scarr, 2000). The use of stomatal frequency analysis in *A. smithii* and *E. obliqua* has not been reported. If stomatal frequencies in *A. melanoxylon*, *A. smithii* and *E. obliqua* are found to be stable, that is, exhibit low intrinsic variation this will make stomatal frequency analysis in these selected species ideal for palaeoclimatological reconstructions, based on stomatal frequency analysis.

The aim of this study is to determine the intrinsic variation associated with stomatal frequency in specimens of *A. melanoxylon*, *A. smithii* and *E. obliqua*, in order to assess their applicability to be applied in the palaeobotanical realm. This will be achieved by examining stomatal frequency for significant variation among leaves, across the leaf surface from base to tip, mid lamina to leaf margin (i.e. within a leaf fragment) and between adaxial (upper) and abaxial (lower) leaf surfaces in leaves of specimens from *A. melanoxylon*, *A. smithii* and *E. obliqua.*

Methods

In order to assess the within- and between-leaf intrinsic variation in stomatal frequency from specimens of *A. melanoxylon*, *A. smithii* and *E. obliqua*, a total number of 20 leaves were collected per species from the outside of the crown behind the branch tip from single specimens in Victoria, Australia (Figure 1). This paper is part of a larger study to examine the potential of stomatal frequency in these species to track increasing subambient [CO_2]; via the use of herbaria. Herbarium-sheets typically only consist of one branch from a

	Acacia melanoxylon		*Acmena smithii*		*Eucalyptus obliqua*	
Test Variable	Within leaf stomatal density (mm2)	Within leaf fragment stomatal density (mm2)	Within leaf stomatal density (mm2)	Within leaf fragment stomatal density (mm2)	Within leaf stomatal density (mm2)	Within leaf fragment stomatal density (mm2)
Leaf	+ (P=0.047)	+ (P=0.011)	– (P=0.148)	+ (P=0.037)	+ (P=0.046)	– (P=0.060)
Location (tip, middle & base)	+ (P=0.019)	– (P=0.285)	– (P=0.191)	– (P=0.896)	– (P=0.331)	– (P=0.617)
Surface	– (P=0.507)	– (P=0.963)	n/a	n/a	+ (P=0.001)	+ (P=0.005)
Leaf*Location	– (P=0.169)	– (P=0.150)	– (P=0.097)	– (P=0.363)	– (P=0.374)	– (P=0.346)
Leaf*Surface	– (P=0.601)	– (P=0.380)	n/a	n/a	+ (P=0.318)	– (P=0.079)
Leaf*Location*Surface	– (P=0.327)	– (P=0.415)	n/a	n/a	– (P=0.242)	– (P=0.313)
	Acacia melanoxylon		*Acmena smithii*		*Eucalyptus obliqua*	
Test Variable	Within leaf stomatal index	Within leaf fragment stomatal index	Within leaf stomatal index	Within leaf fragment stomatal index	Within leaf stomatal index	Within leaf fragment stomatal index
Leaf	– (P=0.417)	– (P=0.098)	– (P=0.220)	+ (P=0.016)	+ (P=0.029)	– (P=0.290)
Location (inner, middle & outer)	– (P=0.067)	– (P=0.424)	– (P=0.174)	– (P=0.205)	– (P=0.973)	– (P=0.418)
Surface	– (P=0.647)	– (P=0.863)	n/a	n/a	+ (P<0.001)	+ (P=0.005)
Leaf*Location	– (P=0.079)	– (P=0.290)	– (P=0.112)	– (P=0.702)	– (P=0.441)	– (P=0.265)
Leaf*Surface	– (P=0.672)	– (P=0.466)	n/a	n/a	– (P=0.517)	– (P=0.223)
Leaf*Location*Surface	– (P=0.613)	– (P=0.734)	n/a	n/a	– (P=0.230)	– (P=0.300)

Table 2: Assessment of intrinsic variation in stomatal density (mm²) and index among leaves (n = 5) from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* using General Linear Model Analysis ('–' - no significant difference; '+' - significant difference, $P \leq 0.05$).

single tree, so the experimental design of this study is to replicate herbaria and assess the intrinsic variation associated with this resource.

Sampled leaves were dried using a plant press and light box over a two-week period. Once dried, 5 leaves were selected randomly from each sample for stomatal frequency analysis. A leaf square approximately 4 x 4 mm was removed from the tip, middle and base of each leaf from each of the three species (Figure 2). Chemical digestion dissolved leaf biological material, leaving the waxy cuticle which was mounted on a microscope slide, ready for stomatal analysis, as per Christophel and Rowett (1996).

Three replicate stomatal / epidermal cell counts were taken using a light microscope fitted with a graded eyepiece on a known viewing field; these counts were obtained from the adaxial and abaxial surface of leaf squares sampled from the tip, middle and base of the 5 leaves of each specimen. Within the leaf fragment i.e. the leaf square removed mid-leaf, 3 replicate stomatal / epidermal cell counts were taken from the inner, middle and outer fragment, on both adaxial and abaxial surfaces. As *A. smithii* is hypostomatous (i.e. stomata restricted to the abaxial surface), no stomatal analysis was conducted between leaf surfaces.

Stomatal density was then expressed as number of stomata per mm², while stomatal index was calculated by equation 1, as per Salisbury (1927).

$$\text{Stomatal Index (S.I.)} = \frac{\text{no. of stomata X 100}}{\text{no. stomata + no. epidermal cells}}$$

(**Equation 1**)

Statistical analysis involved the conduction of a full-factorial repeated-measures general linear model (GLM) analysis (SPSS Version 12.0) to determine significant variation in stomatal frequency, among leaves of the same species, within different positions on the leaf surface, between adaxial and abaxial leaf surfaces, and within a cuticle (i.e. leaf fragment).

Results

Assessment of intrinsic variation in stomatal density from leaves of A. melanoxylon

Significant differences were found in stomatal density amongst different leaves within a single specimen of *A. melanoxylon* and amongst various locations within a single leaf (Table 2). No significant difference was found between adaxial and abaxial leaf surfaces for stomatal density, nor was there any significant interaction between any of the specimens assessed (Table 2).

Variation in stomatal density within a cuticle, a 4 x 4mm leaf square, sampled from the middle of leaves from *A. melanoxylon* were found to be significantly different (Table 2). There was no significant difference in stomatal density sampled from various locations within a leaf fragment, nor was there any difference between leaf surfaces or interactions between specimens assessed (Table 2).

Assessment of intrinsic variation in stomatal index from leaves of A. melanoxylon

Stomatal index of *A. melanoxylon* was found not to vary significantly among different leaves, among locations within the same leaf or between leaf surfaces (Table 2). Variation in stomatal index within a leaf fragment (inner, middle and outer positions), among leaves from the same position and between adaxial and abaxial leaf surfaces were also not significant (Table 2).

Assessment of intrinsic variation in stomatal density from leaves of A. smithii

Stomatal density in *A. smithii* was not significantly different when compared from various locations among leaves (Table 2). Nor was a significant difference noted for stomatal density from different locations within a single leaf, and no interaction between the test subjects was found to be significant (Table 2).

Significant variation was demonstrated in stomatal density within a leaf fragment sampled from the mid point among leaves of *A. smithii* (Table 2). No significant difference was found at various locations within the leaf fragment, nor was there any significant interactions between specimens tested (Table 2).

Assessment of intrinsic variation in stomatal index from leaves of A. smithii

No significant difference was found among leaves when stomatal index was examined from various locations within *A. smithii* leaves (Table 2). Sample position within an individual leaf and interaction between these factors, also did not significantly influence stomatal index (Table 2). When stomatal index was sampled from the mid point among leaves, a significant difference was found (Table 2). Sampling stomatal index from various locations within the leaf fragment revealed no significant variation, nor was there any significant interaction between factors (Table 2).

Assessment of intrinsic variation in stomatal density from leaves of E. obliqua

Stomatal density demonstrated significant variation among leaves and between leaf surfaces from leaves of *E. obliqua*, a significant interaction between leaf * surface test subjects, was also found (Table 2). As the leaves of *E. obliqua* hang vertically, leaf surfaces were designated 1 and 2, as opposed to adaxial (upper) and abaxial (lower) leaf surfaces. No significant difference was noted in stomatal density from different locations within leaves (Table 2). However, stomatal density did vary significantly between leaf surfaces, but there were no significant interactions between any test factors (Table 2).

Assessment of intrinsic variation in stomatal index from leaves of E. obliqua

Intrinsic variation in stomatal index from *E. obliqua* was significant among leaves and between leaf surfaces, no significant interaction was observed between these test factors (Table 2). There was no significant variability in stomatal index in relation to location on the leaf (Table 2). Stomatal index did not vary significantly among leaves when sampled from the same leaf location or among different positions within a leaf fragment, however, a significant difference was found between leaf surfaces (Table 2). Also, interactions between test factors were not significant (Table 2).

Discussion

Intrinsic variation in stomatal density and index was assessed among leaves, within leaves, within leaf fragments and between leaf surfaces from individual specimens of *A. melanoxylon*, *A. smithii* and *E. obliqua*. As hypothesised, stomatal index produced a less variable measure of stomatal frequency than stomatal density (Tables 2), thus being less prone to intrinsic variation, a finding consistent with many studies (Salisbury, 1927; Sharma and Dunn, 1968; James

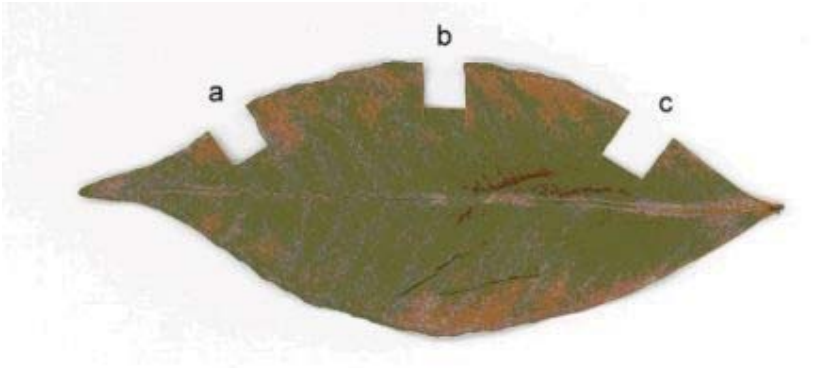

Figure 2: An example of leaf squares removed from a) leaf-tip, b) mid-leaf and c) base-base from a leaf of *Acmena smithii,* which was used for the assessment of within leaf stomatal frequency variation.

and Bell, 1995; Ferris et al., 1996; Smith et al., 1989; Zacchini et al., 1997; Stancato et al., 1999; Royer, 2001). This consistency in stomatal index is due to stomata being expressed as a ratio to epidermal cells, which accounts for epidermal cell expansion that may be influenced by a number environmental variables and hence, alter stomatal density (Salisbury, 1927; Beerling and Chaloner, 1992). Therefore, stomatal index is the preferred measure of stomatal frequency when attempting to apply this character as a biological-proxy (Beerling and Chaloner, 1992; Van Der Burgh et al., 1993).

Intrinsic variation among leaves of Acacia melanoxylon, Acmena smithii and Eucalyptus obliqua

Stomatal density in *A. melanoxylon*, and stomatal density and index in *A. smithii* and *E. obliqua* exhibited significant intrinsic variability among leaves (Tables 2). Interestingly, stomatal density and index in *A. smithii* was not significantly different among leaves when sampled from multiple locations, although was significantly different among leaves when sampled from a consistent location (mid-point). Conversely, in *E. obliqua* stomatal density and index did vary significantly among leaves when sampled from multiple positions, but did not significantly vary when sampled from a static location (Table 2). Either of these scenarios is less than ideal because fossil material may contain complete leaves and / or leaf fragments, so significant variation within any stomatal character has the potential to obscure any possible climatic signal.

Intrinsic variation in stomatal density could be assumed to be attributed to, in part, by leaf-age which would affect epidermal cell expansion and, hence stomatal density (McElwain and Chaloner, 1995). However, this is unlikely as a) all leaves were collected progressively down the branch starting behind any obvious juvenile leaves, and b) stomatal frequency is fixed early in leaf development typically, when the leaf is 10 – 60% of its final size (Tichá, 1982). The most likely cause of intrinsic variation among leaves is shading or genotypic variation.

Shading by self or nearest neighbours would result in a heterogeneous microclimate across the leaf surface, with resultant variations in irradiance, leaf temperature and relative humidity (Givnish and Vermeij, 1976). These variables may potentially influence epidermal cell expansion and stomatal initiation; thus affecting both stomatal density and index (Körner et al., 1983; Ferris et al., 1996; Tichá, 1982; Furukawa, 1997). Interestingly, stomatal index in *A. melanoxylon* did not exhibit any significant intrinsic variation (Table 2), thus whatever factor(s) that influenced stomatal density in *A. melanoxylon*, and both stomatal density and index in *A. smithii* and *E. obliqua* was not influencing stomatal initiation in *A. melanoxylon*.

Intrinsic variation over the leaf surface in Acacia melanoxylon, Acmena smithii and Eucalyptus obliqua

Significant intrinsic variation was observed in stomatal density when sampled from the tip, middle and base of leaves of *A. melanoxylon* (Table 2). Variation in this stomatal character is likely due to microsite variation across the leaf surface, which is driven by boundary layer thickness (Givnish, 1978) and decreased water potential that is, increased evaporative demand and poor water supply (Royer, 2001). The product of increased evaporative demand is smaller epidermal cell size, and increased stomatal density per unit area (Royer, 2001).

No significant variation was found in stomatal index of *A. melanoxylon* among different leaf positions as stomatal index is insensitive to factors that influence epidermal cell size, such as, evaporative demand. Nor was any significant variation observed in stomatal density or index within leaves of *A. smithii* and *E. obliqua* (Tables 2). This lack of intrinsic variation across the leaf suggests that microsite variability over the leaf surface is not large enough to elicit significant change in stomatal initiation or epidermal cell expansion in *A. smithii* and *E. obliqua*, which is in contrast to previous studies (Salisbury, 1927; Sharma and Dunn, 1968; Ferris *et al.*, 1996; Smith *et al.*, 1989; Zacchini *et al.*, 1997; Stancato *et al.*, 1999; Royer, 2001).

Species specific sensitivity to microsite variability over the leaf surface may result from different plant life strategies. For example, *A. melanoxylon* uses phyllodes (modified petioles) as photosynthetic organs due to their high water use efficiency (Brodribb and Hill, 1993) and therefore may potentially be very sensitive to evaporative demand across the leaf surface, in comparison to *A. smithii* and *E. obliqua*.

Intrinsic variation over a leaf fragment in Acacia melanoxylon, Acmena smithii and Eucalyptus obliqua

Examination of the inner, middle and outer regions of a mounted cuticle (i.e. leaf fragment) revealed no significant variation of stomatal frequency in *A. melanoxylon*, *A. smithii* and *E. obliqua* (Table 2). As the inner leaf fragment represents mid lamina and the outer leaf fragment representing the leaf margin, the hypotheses stating that intrinsic variation is expected to be observed across this region of the leaf can be rejected. This finding is in contrast with previous research using other tree species (Salisbury, 1927; Sharma and Dunn, 1969; Smith *et al.*, 1989). Also, this suggests uniform stomatal distribution over leaf fragments in these species and explains the notable lack of intrinsic variation across the leaf surface.

Intrinsic variation between leaf surfaces in Acacia melanoxylon, Acmena smithii and Eucalyptus obliqua

Stomatal frequency did not vary significantly between the adaxial and abaxial leaf surfaces in *A. melanoxylon* (Table 2), suggesting that intrinsic or environmental factor(s) are exerting the same influence upon stomatal

initiation and epidermal cell expansion, irrespective of leaf surface; resulting in consistent stomatal distribution between leaf surfaces. This finding is in contrast to past studies that have found the abaxial surface to be the most stable, in terms of stomatal frequency, differences in these results may be attributed to species-specific variation (Rowson, 1946; Sharma and Dunn, 1968 & 1969; Royer, 2001; Greenwood *et al.*, 2003).

Acmena smithii is hypostomatous, that is, stomata are restricted to the lower leaf surface, so no analysis was conducted for this variable. Significant intrinsic variation was found in stomatal density and index between leaf surfaces of *E. obliqua* (Tables 2), suggesting varying microclimatic conditions between leaf surfaces are eliciting different stomatal frequency responses. The environmental variable that is most likely to be attributed to this response in *E. obliqua* is irradiance. As leaves hang vertically they will receive morning and dusk light directly, one may assume that stomatal frequency would be greater on the leaf surface receiving morning light, as this would be the time when internal leaf [CO_2] is most reduced and thus demand would be at its greatest.

If stomatal frequency is to be employed as a palaeoclimatological tool, it would be highly desirable to select species that 1) have an existing fossil record, and 2) exhibit low degrees of intrinsic variability in their stomatal frequency, thus reducing the error associated with any past climate estimate. Significant intrinsic variation among leaves will result in a larger sample size being required in order to reduce this potential error, which may not always be possible in a limited fossil sample. Also, many fossil leaf samples are fragmented, so using a selected species that does not exhibit intrinsic variation across the leaf surface would be of great use. Stomatal index in *A. melanoxylon* meets all the requirements to be applied in the palaeobotanical realm to provide palaeoclimate estimates, due to the lack of intrinsic variation associated with this stomatal character and an established fossil record. Palaeoclimatological estimates based on leaf morphological characteristics also have the potential to provide valuable information on how future climate change may affect modern-day ecosystems at a regional level. Therefore, the ability to identify the appropriate candidates for palaeoclimatological analysis is fundamental in the interpretation of regional climate change; this research paper identifies an appropriate technique that can be applied for other plant taxa. Future research can extend to other species in the *Acacia*, *Acmena* and *Eucalyptus* genus' to determine if other related species exhibit the same intrinsic variation.

Conclusions

It was found that stomatal index in *A. melanoxylon* was not influenced by any intrinsic factor tested in this study, making stomatal index of *A. melanoxylon* an ideal candidate for application within the palaeobotancial realm.

Stomatal density of *A. melanoxylon* demonstrated significant intrinsic variability among leaves and among different leaf positions. Stomatal density and index in *A. smithii* was only found to vary significantly among leaves, while *E. obliqua* exhibited significant variation in stomatal density and index among leaves, and between leaf surfaces within those leaves.

It can be concluded that stomatal frequency in these three Australian tree species were relatively stable, in that, of the 4 measures of intrinsic variation (1 Among leaves, 2 Over the leaf surface- tip to base, 3 Across the leaf surfaces- mid lamina to margin and 4 Between leaf surfaces) no species exhibited significant variability in more than 2 test variables.

References

- Atchinson, J. and Head, L., 1999. Preparing sub-fossil leaves from *Leporillus* spp. (Stick-nest rat) middens for measuring stomatal parameters. *Quaternary Australasia*, 17, 27-30.
- Atchison, J. M., Head, L. M. and McCarthy, L. P., 2000. Stomatal parameters and atmospheric change since 7500 years before present: Evidence from *Eremophila deserti* (Myoporaceae) leaves from the Flinders Rangers region, South Australia. *Australian Journal of Botany*, 48, 223-232.
- Basinger, J.F., Greenwood, D.R., Wilson, P.G. and Christophel, D.C., 2007. Fossil flowers and fruits of capsular Myrtaceae from the Eocene of South Australia. *Canadian Journal of Botany – Revue Canadienne De Botanique*, 85(2), 204-215.
- Beerling, D.J. and Chaloner, W.G., 1992. Stomatal density as an indicator of atmospheric CO_2 concentration. *Holocene,* 2 (1), 71–78.
- Blackburn, D.T. and Sluiter, I.R.K., 1994. The Oligo-Miocene coal floras of southeastern Australia. In R.S. Hill (editor). *History of Australian Vegetation: Cretaceous to Present.* Cambridge, Great Britain, 433pp.
- Boland, D.J., Brooker, M.I.H., Chippendale, G.M., Hall, N., Hyland, B.P.M., Johnston, R.D., Kleinig, D.A. and Turner, J.D. 1984. *Forest Trees of Australia* (4th Edition). Thomas Nelson, Melbourne, 687pp.
- Brodribb, T. and Hill, R.S., 1993. A Physiological Comparison of Leaves and Phyllodes in *Acacia melanoxylon. Australian Journal of Botany*, 41, 293-305.
- Carpenter, R.A., Hill, R.S., Greenwood, D.R., Partridge, A.D. and Banks, M.A., 2004. No snow in the mountains: Early Eocene plant fossils from Hotham Heights, Victoria, Australia. *Australian Journal of Botany,* 52(6), 685-718.
- Christophel, D.C. and Rowett, A.I., 1996. *Leaf and Cuticles Atlas of Australian Leafy Lauraceae*. Australian Biological Resources Study, Flora of Australia, Supplementary Series 6.
- Ferris, R., Nijs, I., Behaeghe, T. and Impens, I., 1996. Elevated CO_2 and temperature have different effects on leaf anatomy of perennial ryegrass in spring and summer. *Annals of Botany,* 78 (4), 489-497.
- Furukawa, A., 1997. Stomatal frequency of *Quercus myrsinaefolia* grown under different irradiances. *Photosynthetica*, 34 (2), 195-199.
- Givnish, T.J., 1978. Ecological aspects of plant morphology; leaf form in relation to environment. *Acta Biotheoretica*, 27(Supplement), 83-142.
- Givnish, T.J. and Vermeij. G.J., 1976. Sizes and Shapes in Liane Leaves. *American Naturalist,* 110 (975), 743-779.
- Greenwood, D.R., 1992. Taphonomic constraints on foliar physiognomic interpretations of Late Cretaceous and Tertiary palaeoclimates. *Review of Palaeobotany and Palynology*, 71 (1-4), 149-190.
- Greenwood, D.R. and Wing, S.L., 1995. Eocene continental climates and latitudinal gradients. *Geology*, 23(11), 1040-1048.
- Greenwood, D.R., Scarr, M.J. and Christophel, D.C., 2003. Leaf stomatal frequency in the Australian tropical tree *Neolitsea*

dealbata (Lauraceae) as a proxy measure of atmospheric CO_2. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 196 (3-4), 375-393.
- Greenwood, D.R., Wilf, P., Wing, S.L. and Christophel, D.C., 2004. Paleotemperature estimates using leaf margin analysis: Is Australia different? *PALAIOS*, 19(2), 129-142.
- Gullan, P. 1998. *Wild Plants of Victoria*. Viridans Pty Ltd, Melbourne, Victoria.
- Hickey, L.J., 1979. A revised classification of the leaf architecture of dicotyledonous leaves. In C.R. Metcalfe and L. Chalk (editors). *Anatomy of the Dicotyledons*. Clarendon, Oxford, 154pp.
- Hopper, S.D. and Gioia, P., 2004. The Southwest Australian Floristic Region: Evolution and conservation of a global hot spot of biodiversity. *Annual Review of Ecology, Evolution and Systematics,* 35, 623-650.
- James, S.A. and Bell, D.T., 1995. Morphology and anatomy of leaves of *Eucalyptus camaldulensis* clones: variation between geographically separated locations. *Australian Journal of Botany,* 43 (4), 415-433.
- Körner, C., 1988. Does global increase of CO_2 alter stomatal density? *Flora*, 181, 253–257.
- Körner, C., Allison, A. and Hilscher, H., 1983. Altitudinal variation of leaf diffusive conductance and leaf anatomy in heliophytes of montane New Guinea and their interrelation with microclimate. *Flora*, 174, 91-135.
- Kouwenberg, L.L.R., Kurschner, W.M. and Visscher, H., 2004. Changes in stomatal frequency and size during elongation of *Tsuga heterophylla* needles. *Annals of Botany*, 94 (4), 561-569.
- Ladiges, P.Y., Udovicic, F. and Nelson, G., 2003. Australian biogeographical connections and the phylogeny of large genera in the plant family Myrtaceae. *Journal of Biogeography*, 30(7), 989-998.
- McElwain, J.C. and Chaloner, W.G., 1995. Stomatal density and index of fossil plants track atmospheric carbon dioxide in the Palaeozoic. *Annals of Botany*, 76 (4), 389-395.
- Poole, I. and Kurschner, W.M., 1999. Stomatal density and Index: the practice. In T.P. Jones and N.D. Rowe (editors). *Fossil plants and spores: modern techniques.* Geological Society, London. 251-256.
- Poole, I., Lawson, T., Weyers, J.D.B., and Raven, J.A., 2000. Effect of elevated CO_2 on the stomatal distribution and leaf physiology of *Almus glutinosa*. *New Phytologist,* 145 (3), 511-521.
- Poole, I, Weyers, J.D.B., Lawson, T. and Raven, J.A., 1996. Variations in stomatal density and index: implications for palaeoclimatic reconstructions. *Plant, Cell and Environment,* 19 (6), 705-712.
- Rowson, J.M., 1946. The significance of stomatal index as a differential character. Part III. Studies in genera *Atropa, Datura, Digitalis, phytolacca* and in polyploidy leaves. *Quarterly Journal of Pharmacy and Pharmaceuticals,* 19, 136-143.
- Royer, D.L., 2001. Stomatal density and stomatal index as indicators of paleoatmospheric CO_2 concentration. *Review of Palaeobotany and Palynology,* 114 (1-2), 1-28.
- Salisbury, E.J., 1927. On the causes and ecological significance of stomatal frequency with spatial reference to woodland flora. *Philosophical Transactions of the Royal Society of London*, B216, 1-65.
- Scarr, M.J., 2000. The potential of stomatal frequency to be used as a proxy indicator of atmospheric CO_2: using the Victorian temperate forest tree *Acacia melanoxylon*. Proceedings of the Ecological Society of Australia.
- Sharma, G.K. and Dunn, D.B., 1968. Effect of environment on the cuticular features in *Kalanchoe fedschenkoi. Bulletin of the Torrey Botanical Club*, 95 (5), 464-473.
- Sharma, G.K. and Dunn, D.B., 1969. Environmental modifications of leaf surface traits in *Datura stramonium. Canadian Journal of Botany*, 47 (8), 1211-1216.
- Smith, S., Weyers, J.D.B. and Berry, W.G., 1989. Variation in stomatal characteristics over the lower surface of *Commelina communis* leaves. *Plant, Cell and Environment,* 12 (6), 653-659.
- Stancato, G.C., Mazzoni-Viveiros, S.C. and Luchi, A.E., 1999. Stomatal characteristics in different habit forms of Brazilian species of *Epidendrum* (Orchidaceae). *Nordic Journal of Botany,* 19 (3), 271-275.
- Tichá, I. 1982. Photosynthetic characteristics during ontogenesis of leaves: 7. Stomatal density and sizes. *Photosynthetica*, 16 (2), 375-471.
- Uhl, D. and Kerp, H., 2005. Variability of stomatal density and index in the upper Permian conifer *Quadrocladus* Mädler- a taphonomic case study. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 218 (3-4), 205-215.
- Van Der Burgh, J., Visscher H., Dilcher, D.L. and Kürschner, W.M., 1993. Paleoatmospheric signatures in Neogene fossil leaves. *Science*, 260, 1788-1790.
- Wagner, F., Dilcher, D.L. and Visscher, H., 2005. Stomatal frequency responses in hardwood-swamp vegetation from Florida during a 60-year continuos CO_2 increase. *American Journal of Botany*, 92 (4), 690-695.
- Woodcock, D.W., 1992. Climate reconstruction based on biological indicators. *Quaternary Review of Biology*, 67 (4), 457-477.
- Wing, S.L. & Greenwood, D.R. 1993. Fossils and fossil climates: the case for equable Eocene continental interiors. *Philosophical Transactions of the Royal Society of London,* B341, 243-252.
- Zacchini, M., Morini, S. and Vitagliano, C., 1997. Effects of photoperiod on some stomatal characteristics of an *in vitro* cultured fruit tree shoots. *Plant, Cell, Tissue and Organ Culture,* 49 (3), 195-200.