1 # Is pollen size a robust proxy for moisture availability?

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15

16    **Abstract**

17    The development of well-constrained palaeo-proxies that enable the reconstruction of past

18    climate change is becoming an ever more important field of scientific enquiry within the

19    palaeobotanical community, with the potential to deliver broader impacts linked to

20    understanding of future anthropogenic climate change. One of the major uncertainties in

21    predicting climate change is how the hydrological cycle will respond to future warming.

22    Griener and Warny (2015, Review of Palaeobotany and Palynology 221, 138-143) suggested

23    that pollen size might be a useful proxy for tracking moisture availability, as pollen size

24    appears to be negatively correlated with moisture. Given the long fossil record of pollen and

25    spores such a proxy would have broad scope and the potential to deliver much needed

26    information. Here we set out to fully evaluate and test the robustness of this proxy. We focus

27    on a number of a key issues: controls on pollen size, data analysis, and finally proxy

28    validation. Using this approach we find that there is little theoretical or empirical support for

29    the original relationship proposed by Griener and Warny. Consequently it is currently

30    premature to use pollen size as a moisture availability proxy in the fossil record. However,

31    we recognise that the technique may have potential and conclude by offering a series of

32    recommendations that would rigorously assess and test for a relationship between pollen size

33    and moisture availability.

34

## 1. Introduction

In the absence of direct measurements of climate in Earth's past, palaeoclimate proxies have become essential for reconstructing climatic trends and ground-truthing climate models (Masson-Delmotte et al., 2013). However before these proxies can be deployed they need to be fully tested to assess both accuracy and the precession that underlie their predictive elements (Lomax and Fraser, 2015; Lomax et al., 2012). While fossil pollen and spores have traditionally been used in a passive manner (Jardine et al., 2016) to infer past climates based on the climatic tolerances of their nearest living extant relatives (e.g. Mosbrugger and Utescher, 1997; Utescher et al., 2014), there have also been attempts to use palynological morphological (Griener and Warny, 2015; Kürschner et al., 2013) and chemical data (Fraser et al., 2014; Fraser et al., 2011; Jardine et al., 2017; Jardine et al., 2016; Lomax and Fraser, 2015; Lomax et al., 2008; Rozema et al., 2009; Rozema et al., 2001a; Rozema et al., 2001b; Watson et al., 2007) to reconstruct climatic parameters more directly.

Griener and Warny (2015), hereafter G&W15, introduced a proxy for moisture availability based on the size of pollen grains. This proxy is centered on the idea that larger pollen grains will be relatively more resistant to desiccation stress, because of a decreased surface area to volume ratio (Ejsmond et al., 2011; Griener and Warny, 2015). There should therefore be a negative correlation between moisture availability and pollen size at certain taxonomic levels (i.e. within species, genera or families). Moisture availability is a key climatic parameter (Wilf et al., 1998) and an important control on plant distributions (Engelbrecht et al., 2007; Gentry, 1988). A new, robust proxy for moisture availability would therefore be a particularly valuable tool for the scientific community. G&W15 demonstrated this proxy by relating the size of modern *Nothofagus* pollen grains to mean annual precipitation (MAP), and then reconstructing size trends in Antarctic *Nothofagidites*

63 *lachlaniae*-complex pollen grains from the Eocene to the Miocene. Partial validation of the

64 fossil pollen size data was carried out via a descriptive (i.e. non-statistical) comparison to the

65 intrinsic water use efficiency ($WUE_i$) of Eocene *Nothofagus*, derived from the carbon isotope

66 discrimination (Δ) of *Nothofagidites* sporopollenin (Griener et al., 2013).

67

68        Here, we focus on a number of key issues that we have identified in the theoretical

69 basis and validation of the moisture availability proxy of G&W15. Specifically, we focus on

70 three aspects: first, known controls on pollen size; second, the data analysis techniques

71 employed by G&W15 for validating their modern pollen size data, including a lack of

72 accounting for phylogenetic autocorrelation; and third, the lack of supporting evidence for the

73 palaeo-moisture availability reconstruction developed by G&W15 in coeval fossil data.

74 Finally, we suggest some ways in which this proxy may be more fully developed and

75 validated.

76

77 **2. Controls on pollen size**

78 *2.1 Genome size*

79        There is extensive literature showing that pollen size in extant plants is related to

80 genome size and that this relationship scales with the level of ploidy (Bennett, 1972; De

81 Storme et al., 2013). For example Bennett (1972) showed that there is a highly significant

82 positive relationship between pollen volume and genome size in the grasses (y = 2.1 +

83 0.643*x, $r^2 = 0.91$, $p <0.001$, $n = 15$). Although these data are not corrected for the effects of

84 phylogeny (see section 3, below) it does highlight that factors other than moisture availability

85 have the capacity to drive changes in pollen size and that these factors can explain a greater

86 proportion of the variance. A full investigation of the relationship between pollen diameter

87 and genome size indicates that relationship appears to hold within disparate plant groups

88   especially when looking at ploidy but does not scale across broad groups when phylogeny is

89   accounted for (Knight et al., 2010). These findings suggest that there is a strong phylogenetic

90   signal (see section 3) that links pollen size to genome size and that is independent of

91   environment.

92

93        There is some evidence for polyploidy being a selective advantage in dryer

94   environments. The larger cells of polyploids can lead to higher xylem hydraulic conductance,

95   for example (Thompson et al., 2015), as well as differences in stomatal apparatus

96   (Manzaneda et al., 2012). Manzaneda et al. (2012) found that aridity is a strong predictor of

97   ploidy level in the temperate grass *Brachypodium distachyon*, with tetraploid individuals

98   having higher water use efficiency and increased tolerance to drought relative to diploid

99   individuals. Further support for polyploidy conferring a selective advantage with regard to

100  water stress comes from Garbutt and Bazzaz (1983), Watanabe (1986) and Li et al. (1996),

101  all of whom demonstrated that polyploid plants are more tolerant of water stress than

102  diploids. However, there is also evidence for polyploid plants being less well adapted to

103  drought conditions (Baldwin, 1941).  Similar dichotomous results are also found when

104  comparing polyploid plants to diploids when looking for trends that confer cold tolerance and

105  shade tolerance (see Maherali et al., 2009 for details). Taken together these findings suggest

106  that while there is some tentative support for a general relationship between pollen size and

107  moisture availability (Ejsmond et al., 2011; Griener and Warny, 2015), it is highly likely that

108  this response would be mediated through genome size, rather than being a direct cause and

109  effect relationship. Consequently, any other factors influencing the relationship between

110  ploidy/genome size and habitat preference would bias the G&W15 moisture availability

111  proxy, limiting the inferences that could be made from it.

112

*2.2 Palynological processing methods, mounting media and taphonomy*

It has long been known that different palynological processing methods can modify pollen size (Christensen, 1946; Faegri and Iversen, 1975; Moore et al., 1991; Reitsma, 1969), although this was not directly discussed by G&W15, either in relation to their own samples or as implications for future moisture availability reconstructions. This is particularly important because G&W15 treated their modern and fossil samples differently, with no processing for the modern samples and a standard processing protocol of HF and HCl for the fossil samples. HF has been shown to decrease the size of pollen grains (Faegri and Iversen, 1975; Moore et al., 1991; Schüler and Behling, 2011), meaning that the size changes demonstrated by G&W15 may not be directly comparable across datasets. Since no pollen size-MAP calibration or quantitative precipitation estimates were attempted by G&W15 this in itself does not compromise their results, but it will need to be considered if this proxy is developed further as a quantitative technique. Other processing methods not carried out by G&W15, such as treatment with KOH and acetolysis, have been reported to cause size changes in palynomorphs (Christensen, 1946; Faegri and Iversen, 1975; Moore et al., 1991; Reitsma, 1969), so as with HF care will need to be taken when analysing grain size trends across sample sets that have undergone different processing protocols.

The choice of storage and mounting media is also known to have an impact on pollen size (Andersen, 1960; Christensen, 1946; Cushing, 1961; Faegri and Iversen, 1975; Moore et al., 1991; Reitsma, 1969; Sluyter, 1997). G&W15 mounted their modern and fossil samples in glycerine jelly, which can cause swelling of pollen grains, either through absorption of water from the atmosphere (Christensen, 1946; Moore et al., 1991) or softening of the exine and subsequent deformation caused by pressure from the microscope coverslip (Cushing, 1961). G&W15 did not state how long their samples were stored in glycerine jelly prior to

138    analysis, so it is not possible to say how much of an impact it may have had on their reported

139    measurements. Clearly any pollen size analyses that make use of samples that have been

140    stored in glycerine jelly will need to take possible size changes into account, especially if

141    different batches of samples were processed at different times. An alternative storage and

142    mounting medium such as silicone oil (Andersen, 1960; Sluyter, 1997) may be a better option

143    for pollen size measurements, or using coverslip supports (e.g. sand grains or splints from

144    other coverslips) to limit the downward pressure on the pollen grains (Cushing, 1961).

145

146        Finally, taphonomic processes will impact upon the size and shape of fossil

147    palynomorphs. Damage to pollen grains and spores through folding, pinching or breaking is a

148    common occurrence in fossil palynological samples (Havinga, 1967; Mander et al, 2012;

149    Tweddle and Edwards, 2010; Twiddle and Bunting, 2010), and makes consistent

150    measurements challenging, especially across changes in taphonomic regimes or in

151    comparison to modern specimens. Careful quality control will therefore be needed when

152    selecting specimens for measurement, which may limit the broader utility of the proxy unless

153    the target taxon is abundant and well-preserved through the time period of interest. Taken

154    together, these various factors show that care needs to be taken when selecting and

155    processing samples for pollen size analysis, and that all processing protocols need to be fully

156    reported in the literature. While these specific issues may not have compromised the analysis

157    in G&W15, they will need to be carefully considered and taken into account in any further

158    research on pollen size-based proxies, whether these relate to moisture availability or genome

159    size in the fossil record.

160

161    **3. Analysis of data**

162    The modern validation dataset of G&W15 comprised 157 measurements of

163    *Nothofagus* pollen diameters taken from 19 herbarium specimens representing 12 species (not

164    13, as stated in G&W15), with between 1 and 30 grains measured per specimen. G&W15

165    tested the relationship between pollen size and mean annual precipitation using ordinary least

166    squares (OLS) linear regression, fitting a linear model though the mean values for each

167    sample. OLS regressions were carried out for the whole dataset together (Fig. 1 in G&W15)

168    and separately for the subgenera *Brassospora*, *Fuscospora* and *Nothofagus* (Fig. S1 in

169    G&W15). The subgenus *Lophozonia* is only represented by one measurement in the G&W15

170    dataset, from the species *N. obliqua*, and so could not be modelled separately.

171

172    One of the underlying assumptions of OLS regression is that the values in the

173    response variable are independent and identically distributed (i.i.d.) (Rohlf, 2006; Zuur et al.,

174    2009). Data from a range of taxa are never truly independent, because the taxa will be in

175    some way descended from a common ancestor, with more closely related species being more

176    similar than distantly related species (Garland and Ives, 2000). This phylogenetic signal in

177    the response variable violates the assumption of independence (Garland and Ives, 2000;

178    Martins and Hansen, 1997; Rohlf, 2006), inflating the Type 1 error rate (i.e. incorrectly

179    rejecting a true null hypothesis, and finding a statistically significant result where none is

180    present) (Rohlf, 2006).

181

182    Several methods have been developed to account for the phylogenetic signal within

183    biological trait data (Cooper et al., 2016). One of the most commonly used is phylogenetic

184    independent contrasts (PIC), which calculates differences (contrasts) between the

185    character/trait values of pairs of sister taxa across the tips and nodes of a phylogeny

186    (Felsenstein, 1985; Garland et al., 1992; Garland and Ives, 2000). PIC implicitly assumes a

187    Brownian motion model of trait evolution, i.e. an evolutionary random walk through trait

188    space (Cooper et al., 2016; Garland and Ives, 2000). The calculated contrasts are

189    phylogenetically independent and can be used in standard statistical analyses such as

190    correlations, allowing for the role of phylogeny in driving the relationship to be assessed. For

191    example from a palaeobotanical perspective PIC has been used to explore if carbon isotope

192    fractionation is driven by phylogeny (Lomax et al., 2012) and to determine that within

193    angiosperms the relationship between genome size and guard cell length is independent of

194    phylogeny (Beaulieu et al., 2008; Lomax et al., 2014), enabling broad scale reconstruction of

195    the genome size of fossil plants (Lomax et al., 2014).

196

197        More recently, phylogenetic generalised least squares (P-GLS) regression has been

198    developed, which allows for phylogenetic relatedness to be directly modelled as a correlation

199    structure within the linear regression framework (Blomberg et al., 2012; Garland and Ives,

200    2000; Grafen, 1989; Martins and Hansen, 1997). P-GLS regression has the advantages that (i)

201    it is more flexible than PIC, allowing for different underlying models of trait evolution

202    (Rohlf, 2006), (ii) the variables can be modeled directly, rather than as contrasts (Garland and

203    Ives, 2000), and (iii) it can be readily extended to more complex mixed effects models

204    (Blomberg et al., 2012). However when a Brownian motion model of trait evolution is

205    assumed in P-GLS regression the two techniques give identical results (Blomberg et al.,

206    2012; Garland and Ives, 2000; Rohlf, 2006).

207

208        Here, we re-analyse the G&W15 dataset using P-GLS. G&W15 made their dataset

209    (specimen pollen size mean and standard deviation, and associated MAP) available in Table

210    S1 of their paper. We use the *Nothofagus* molecular phylogeny of Sauquet et al. (2012),

211    downloaded from TreeBASE (www.treebase.org) on 18/01/2017, to represent the

212   phylogenetic relatedness among taxa. The phylogeny comprises 27 *Nothofagus* species (Fig

213   1), as well as 21 outgroup species from the core Fagales, and so was first trimmed down to

214   the taxa represented in the G&W15 dataset. *Nothofagus rubra* and *Nothofagus starkenborghii*

215   are not present in the molecular phylogeny, and so were removed from the G&W15 dataset.

216   The pollen size and MAP means for the remaining 10 taxa were then calculated for use in the

217   P-GLS regression. We used a simple Brownian motion model of trait evolution across the

218   trimmed *Nothofagus* phylogeny as a correlation structure in the P-GLS regression. Data

219   analysis was carried out in R v. 3.3.1 (Team, 2016) using the packages ape v. 4.1 (Paradis et

220   al., 2004), phytools v. 0.5-64 (Revell, 2012), nlme v. 3.1-131 (Pinheiro et al., 2017) and

221   astrochron v. 0.6.5 (Meyers, 2014).

222

223        Mapping pollen size directly onto the molecular phylogeny using ancestral character

224   estimation (Revell, 2013) shows a degree of phylogenetic structuring (i.e. similar values in

225   the sister taxa *N. carrii* and *N. grandis*, and *N. dombeyi* and *N. betuloides*), confirming the

226   importance of correcting for phylogenetic non-independence in the regression model (Fig 2a;

227   see also Fernández et al., 2016). Regressing pollen size onto MAP (pollen size is the response

228   or dependent variable, MAP is the explanatory or independent variable) using P-GLS

229   regression gives the model y = 35.2 - 0.003*x, $p = 0.003$, $n = 10$, whereas an OLS regression

230   of the same version of the dataset gives the model y = 38.4 - 0.005*x, $p = 0.0006$, $n = 10$ (Fig

231   2b). While both model fits are statistically significant, the $p$ value is an order of magnitude

232   lower in the P-GLS model, and the slope is also shallower. The different slopes between the

233   two models shows the importance of including phylogeny for any regression models being

234   used for calibration and quantitative moisture availability reconstructions.

235

236    There is also the more general point that the G&W15 dataset itself is quite small, with

237    limited replication of individual plants within each species. Seven of the species in the dataset

238    are represented by just one herbarium specimen, three by two specimens, and two by three

239    specimens. This not only makes it challenging to look at within-species variation, to

240    investigate whether populations at different MAP levels exhibit differences in pollen size, but

241    also makes it impossible to assess whether non-significant relationships at the subgenus level

242    are due to a genuine lack of a response to moisture availability or insufficient data. G&W15

243    carried out regressions for the three subgenera with more than one species represented, and

244    only found a significant relationship for one of them, *Brassospora* (Fig. 3, note that the

245    subgenus *Nothofagus* was reported as significant but had a *p* value of <0.17 in G&W15). The

246    significant relationship for *Brassospora* is largely driven by one outlier, the species *N.*

247    *discoidea*, which with a mean grain size of 32.1 µm is considerably larger than the other

248    *Brassospora* species sampled (Figs. 3 and 2a). Removing the *N. discoidea* data point from the

249    dataset and re-running the regression reduces the slope of the fitted model from -0.006 to -

250    0.003, the $r^2$ from 0.73 to 0.28, and the *p* value from 0.003 to 0.18 (i.e. the relationship is no

251    longer statistically significant without this one species). There is therefore limited evidence

252    for a consistent, statistically robust relationship between pollen size and MAP below the level

253    of genus. While this is very possibly down to the small sample sizes involved, this in itself

254    suggests that the dataset used by G&W15 is too limited to robustly validate this proxy, and

255    that further replication across taxonomic ranks is needed if it is to be confidently deployed as

256    a palaeoclimate proxy.

257

258    **4. Lack of evidence in fossil data**

259    Past moisture availability reconstructions based on fossil pollen size should show

260    good agreement with other moisture availability or precipitation proxies (assuming these

261    themselves are broadly reliable). G&W15 measured *Nothofagidites lachlaniae*-complex

262    pollen size from the Eocene to the Miocene of the Antarctic Peninsula (data available in

263    Table S2 of G&W15), and interpreted changes in the context of moisture availability. Griener

264    et al. (2013) generated a moisture availability proxy record for the late Eocene portion of the

265    same cores, based on *Nothofagidites* pollen $\delta^{13}$C discrimination. The $\Delta^{13}$C record shows an

266    overall decrease through time, suggesting a decrease in moisture availability, and thus we

267    would expect this to be reflected in a trend towards larger pollen sizes (i.e. the two records

268    should show a negative correlation).

269

270        The decrease in moisture availability suggested by the $\Delta^{13}$C dataset is less clear in the

271    pollen size dataset, however, especially given the large errors on the pollen size means (Fig.

272    4a). Interpolating the $\Delta^{13}$C data to the same depth levels as the size data, and regressing size

273    onto $\Delta^{13}$C with OSL regression gives the model y = 33.40 - 0.48*x, $r^2 = 0.08$, $p = 0.37$, $n =$

274    12 (Fig. 4b). Therefore the regression reveals the expected negative correlation, but the

275    relationship is not statistically significant and the $\Delta^{13}$C data only explains a small proportion

276    of the variance in the pollen size data. The negative correlation between pollen size and $\Delta^{13}$C

277    is also largely driven by one extreme value (Fig. 4b). Given that the size and discrimination

278    data have been developed on the same plant group from the same sedimentary record this

279    result offers no support for a link between moisture availability and pollen size. This proxy

280    therefore cannot be successfully validated in the fossil record, although we acknowledge that

281    other factors that drive stomatal closure (such as elevated $CO_2$) which control discrimination

282    could also influence $\Delta^{13}$C. However experimental work has shown that water availability

283    does act as a primary control on $\Delta^{13}$C even over a range of $CO_2$ concentrations relevant to the

284    geological record (Lomax et al., 2012).

285

**5. Suggestions for further proxy development**

For the reasons outlined above, we feel that it is currently premature to use pollen size as a moisture availability proxy in the fossil record. In order to fully test and validate this as a robust proxy, we suggest that the following measures are needed:

1. A better understanding of the relationship(s) between genome size, abiotic factors including moisture availability and pollen size.

2. Considerably more modern pollen size data, with greater replication across taxonomic ranks, to robustly quantify the relationship between pollen size and moisture availability. Analysis of these data will require a proper accounting for phylogenetic dependency, and also any spatial autocorrelation between sampling localities (Zuur et al., 2009).

3. Data from controlled growth experiments, with pollen harvested from plants grown under a range of environmental conditions, to better understand the external controls on pollen size within and among species.

4. Further palaeo-palynological datasets where there are existing precipitation indicators, to allow for validation in the fossil record. Quaternary pollen records may be best suited to this, because of better-constrained environmental conditions compared to the deep time fossil record.

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**Figure legends**

**Fig. 1**. *Nothofagus* phylogeny of Sauquet et al. (2012), with the taxa in the G&W15 dataset in bold type.

**Fig. 2**. A) *Nothofagus* phylogeny of Sauquet et al. (2012) limited to the taxa in the G&W15 dataset, with pollen size mapped on using ancestral character estimation (Revell, 2013). B) Pollen size plotted against mean annual precipitation, showing the mean values for each species. Dashed line = ordinary least squares (OSL) model, solid line = phylogenetic generalised least squares (P-GLS) model.

**Fig. 3.** *Nothofagus* pollen size from G&W15 plotted against mean annual precipitation, showing the mean values for each specimen and OSL regression fits for each subgenus (solid lines are fitted models, dashed lines are 95% confidence intervals). Individual regression models are: *Brassospora*, $y = 39.90 - 0.006*x$, $r^2 = 0.73$, $p = 0.003$, $n = 9$; *Fuscospora*, $y = 31.55 - 0.002*x$, $r^2 = 0.37$, $p = 0.28$, $n = 5$; *Nothofagus*, $y = 35.47 - 0.005*x$, $r^2 = 0.89$, $p = 0.06$, $n = 4$.

**Fig. 4**. A) Eocene *Nothofagidites* pollen size and $\Delta^{13}C$ data for the SHALDRIL 3C core. Black points and line = $\Delta^{13}C$ data from Griener et al. (2013). Grey points with error bars = pollen size data from 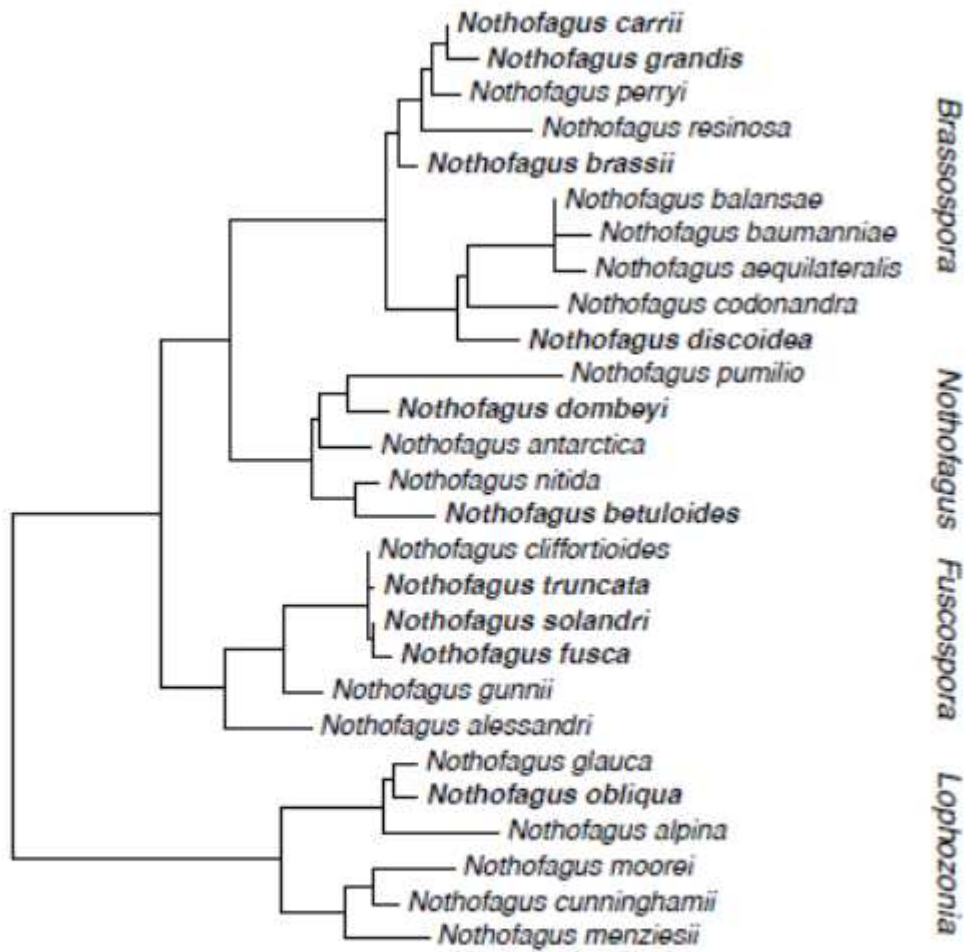G&W15, points are sample means and error bars are 1 standard deviation. B) Eocene *Nothofagidites* pollen size regressed against *Nothofagidites* $\Delta^{13}C$, with the $\Delta^{13}C$ data interpolated to the same sampling depths as the pollen size data. Black solid line is the fitted regression model, dashed lines are 95% confidence intervals.
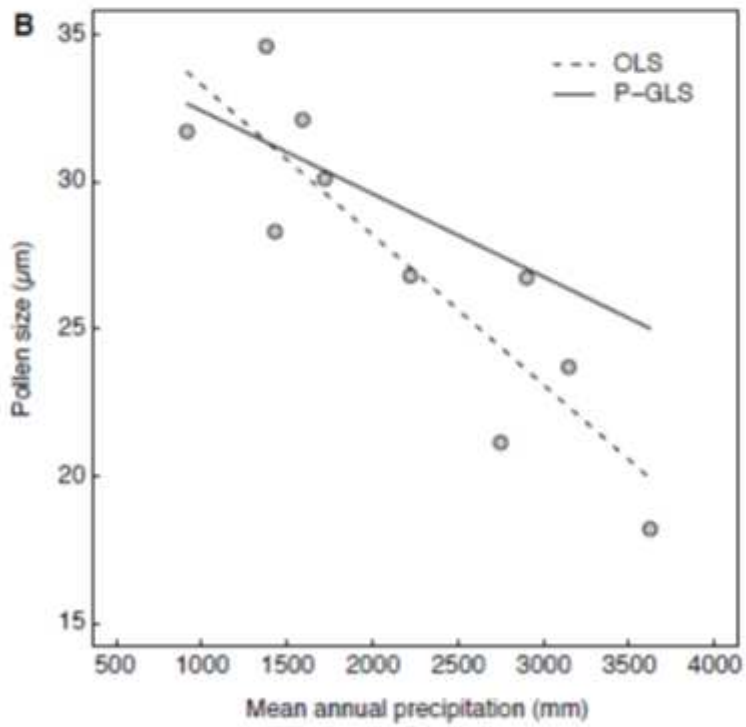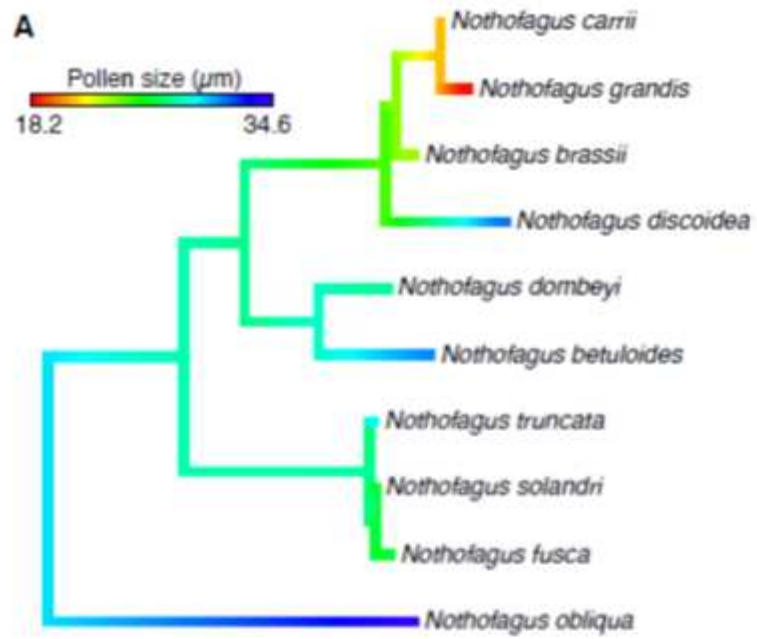
Jardine and Lomax
Is pollen size a robust proxy for moisture availability?
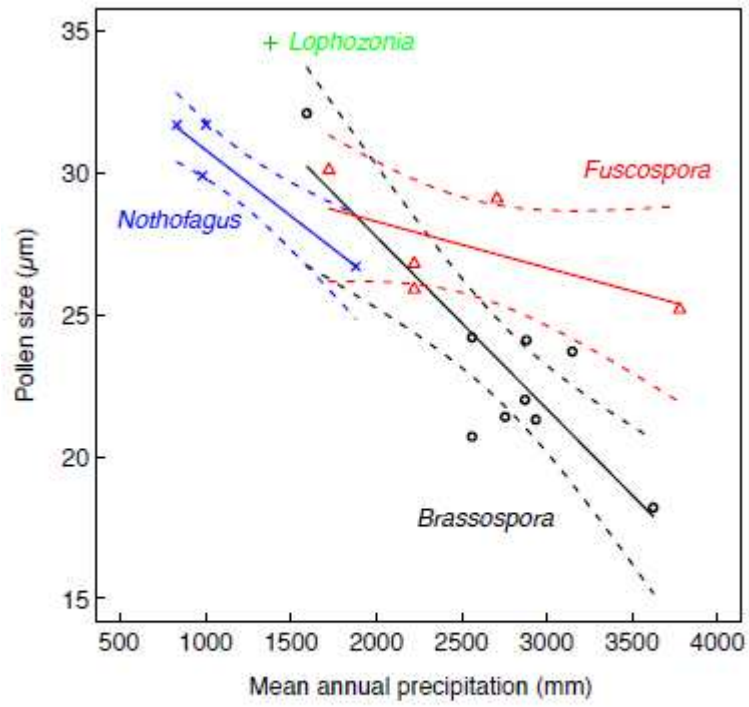Figure 1

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Jardine and Lomax
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Figure 2

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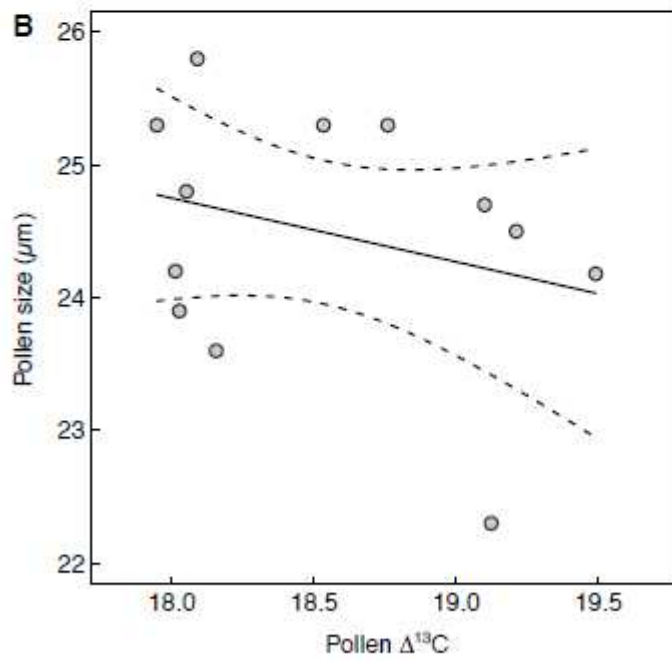Is pollen size a robust proxy for moisture availability?
Figure 3
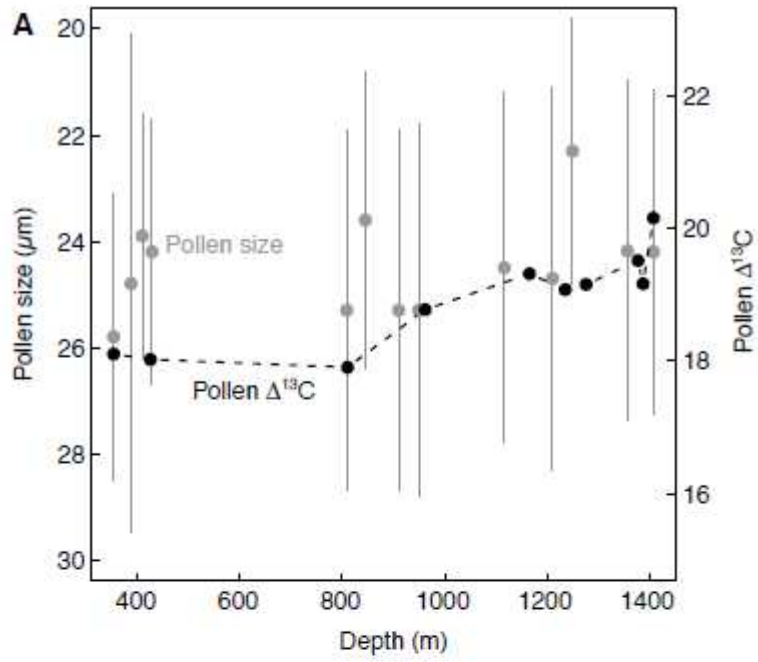
Jardine and Lomax
Is pollen size a robust proxy for moisture availability?
Figure 4

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