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A new positive relationship between pCO_2 and stomatal frequency in *Quercus guyavifolia* (Fagaceae): a potential proxy for palaeo-CO_2 levels

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• **Background and Aims** The inverse relationship between atmospheric CO_2 partial pressure (pCO_2) and stomatal frequency in many species of plants has been widely used to estimate palaeoatmospheric CO_2 (palaeo-CO_2) levels; however, the results obtained have been quite variable. This study attempts to find a potential new proxy for palaeo-CO_2 levels by analysing stomatal frequency in *Quercus guyavifolia* (*Q. guajavifolia*, Fagaceae), an extant dominant species of sclerophyllous forests in the Himalayas with abundant fossil relatives.
• **Methods** Stomatal frequency was analysed for extant samples of *Q. guyavifolia* collected from17 field sites at altitudes ranging between 2493 and 4497 m. Herbarium specimens collected between 1926 and 2011 were also examined. Correlations of pCO_2–stomatal frequency were determined using samples from both sources, and these were then applied to *Q. preguyavaefolia* fossils in order to estimate palaeo-CO_2 concentrations for two late-Pliocene floras in south-western China.
• **Key Results** In contrast to the negative correlations detected for most other species that have been studied, a positive correlation between pCO_2 and stomatal frequency was determined in *Q. guyavifolia* sampled from both extant field collections and historical herbarium specimens. Palaeo-CO_2 concentrations were estimated to be approx. 180–240 ppm in the late Pliocene, which is consistent with most other previous estimates.
• **Conclusions** A new positive relationship between pCO_2 and stomatal frequency in *Q. guyavifolia* is presented, which can be applied to the fossils closely related to this species that are widely distributed in the late-Cenozoic strata in order to estimate palaeo-CO_2 concentrations. The results show that it is valid to use a positive relationship to estimate palaeo-CO_2 concentrations, and the study adds to the variety of stomatal density/index relationships that available for estimating pCO_2. The physiological mechanisms underlying this positive response are unclear, however, and require further research.

Key words: Stomatal density, stomatal index, atmospheric CO_2 concentration, palaeo-CO_2 reconstruction, altitudinal gradient, historical specimen, climate change, oak, *Quercus guyavifolia*, *Q. guajavifolia*.

INTRODUCTION

Palaeoatmospheric CO_2 (palaeo-CO_2) concentration estimates provide important palaeoenvironmental information in geological time and a baseline reference to understand future climatic change. Atmospheric CO_2 concentration has been hypothesized to be a primary determinant of global climate change; periods of low atmospheric CO_2 concentrations witnessed major glaciations, whereas those with higher CO_2 concentrations had warmer conditions (Retallack, 2001; Kürschner *et al*., 2008; Lunt *et al*., 2008; Lacis *et al*., 2010; Smith *et al*., 2010). This CO_2–temperature relationship is conspicuous during the Quaternary and has also been confirmed for other time periods: for example, the Paleocene–Eocene thermal maximum (PETM) was a brief but intense interval of global warming associated with elevated atmospheric CO_2 concentration (Zachos *et al*., 2005). In addition, CO_2 levels play a crucial role in affecting the ecology and physiology of plants.

To understand the relationship of CO_2, climate change and ecological function of CO_2, many attempts have been made to estimate palaeo-CO_2 throughout the Phanerozoic (Pagani *et al*., 1999; Pearson and Palmer, 2000; Berner and Kothavala, 2001; Berner, 2006; Tripati *et al*., 2009; Seki *et al*., 2010). A comparatively reliable method is to measure the CO_2 composition of air locked in glacial ice (Petit *et al*., 1999; Lüthi *et al*., 2008). However, this method can only be applied for the past 800 000 years because of the absence of older glacial ice (Lüthi *et al*., 2008). Pre-ice core CO_2 concentration estimations rely on numerous independent palaeobotanical and geochemical proxies and biogeochemical models derived from palaeobotanical and geochemical proxies, such as geochemical models (Berner and

Kothavala, 2001; Berner, 2006), the $\delta^{13}C$ in palaeosols (Ekart *et al*., 1999; Myers *et al*., 2012), $\delta^{13}C$ of the organic remains of phytoplankton (Seki *et al*., 2010), the $\delta^{11}B$ and B/Ca ratio of marine carbonate (Pearson and Palmer, 2000; Tripati *et al*., 2009; Seki *et al*., 2010) and stomatal frequency [expressed as stomatal density (SD) or stomatal index (SI)] in fossil leaves (e.g. Royer *et al*., 2001; Kürschner *et al*., 2008). Among these approaches, the $\delta^{13}C$ in palaeosols method and the stomatal frequency method are terrestrial-based proxies, and the others are marine-based proxies. However, estimates of the palaeo-CO_2 concentration made using these different approaches are quite variable (Royer *et al*., 2001*a*; Beerling and Royer, 2011). Thus, more research efforts focused on a single time period using different proxies is required. This is especially true for the mid-Miocene climatic optimum [18–15 million years ago (Ma)] and the middle to late Pliocene (3·6–2·6 Ma) (Beerling and Royer, 2011) because both of them were globally warm and relatively recent and may be comparable with the Earth's immediate future with increasing greenhouse gases. Fortunately, there are abundant fossils from these two time periods (van der Burgh *et al*., 1993; Kürschner *et al*., 1996; Kürschner *et al*., 2008; Retallack, 2009; Stults *et al*., 2011) and we also found many oak fossils, providing an ideal opportunity to study the CO_2–temperature relationship during these warm climate intervals by estimating the palaeo-CO_2 concentration using the stomatal frequency method.

Generally, the stomatal frequency method is based on the inverse correlation between atmospheric CO_2 partial pressure (pCO_2) and leaf stomatal frequency which is species specific and observed in many C_3 plants (Woodward, 1987; Kürschner *et al*., 2001; Royer, 2001; Beerling and Royer, 2002*a*; Kouwenberg *et al*., 2003). The method has been widely used to estimate palaeo-CO_2 levels by applying the correlation to closely related plant fossils. Numerous genera and species have been used, such as *Ginkgo* (Retallack, 2001; Royer *et al*., 2001*b*; Beerling and Royer, 2002*a*; Retallack, 2009; Smith *et al*., 2010), *Metasequoia* (Royer *et al*., 2001*b*; Doria *et al*., 2011), other conifers (Passalia, 2009; Steinthorsdottir and Vajda, 2013), cycads (McElwain *et al*., 1999; Haworth *et al*., 2011*b*), *Quercus* (van der Burgh *et al*., 1993; Kürschner *et al*., 1996), Lauraceae (McElwain, 1998; Greenwood *et al*., 2003; Kürschner *et al*., 2008) and *Betula* (Finsinger and Wagner-Cremer, 2009). The precision of identifying nearest living relatives (NLRs) of fossil species and the accuracy of the correlation between stomatal frequency and pCO_2 can have profound effects on the estimates because the relationship is species specific. Royer (2001) summarized the stomatal frequency of 176 previously published C_3 plant species and showed that a majority of the species had inverse correlations; some had no significant relationship; only $\leq$12 % had a positive correlation; and species from the same genus may have inconsistent relationships (McElwain *et al*., 1995; Rundgren and Beerling, 1999; Eide and Birks, 2004; Finsinger and Wagner-Cremer, 2009; Haworth *et al*., 2010*b*). Almost all the previous studies have been based on an inverse relationship between atmospheric pCO_2 and stomatal frequency to estimate palaeo-CO_2 levels. Recently, a positive relationship between atmospheric pCO_2 and stomatal frequency in *Typha orientalis* was used to estimate palaeo-CO_2 levels during the Plio-Pleistocene transition (Bai *et al*., 2014). These studies indicate that it is essential to establish the specific stomatal frequency–pCO_2 relationship of a fossil's NLR before using the relationship to estimate palaeo-CO_2 levels. To achieve this, many studies have used three primary approaches to investigate the response of stomatal frequency to CO_2 variation: (1) study leaves collected over an extended period of time because atmospheric CO_2 concentration has increased from approx. 280 to 390 ppm over the last 150 years; (2) study leaves from different locations along an altitudinal gradient because atmospheric pCO_2 declines as barometric pressure decreases with increasing altitude; and (3) greenhouse experiments in which stomatal frequency can be counted in response to elevated CO_2 concentrations. The first and third approaches have been widely used (Woodward, 1987; van der Burgh *et al*., 1993; Royer *et al*., 2001*b*; Greenwood *et al*., 2003; Kouwenberg *et al*., 2003; Haworth *et al*., 2011*a*), although the first approach may be somewhat constrained by the availability of historical specimens. The third approach misrepresents the potential for proportional population changes within a gene pool, and it does not consider taxonomic differences in plant generation times (Royer, 2001), and thus may fail to reflect long-term, genetic responses to slow changes in the environment of plants (Woodward, 1988; Beerling and Chaloner, 1993; McElwain and Chaloner, 1995). The second method has been used less frequently (McElwain, 2004; Kouwenberg *et al*., 2007) because of the difficulty of collecting one species over a long altitudinal gradient.

Oak plants of *Quercus guyavifolia* H. Lév. [= *Q. pannosa* Hand.-Mazz. (Flora of China), *Q. guajavifolia* H. Lév. (Flora of China, Volume 4, page 375)] is a dominant species in the sclerophyllous forests along the steep altitudinal gradients in the Qinghai-Tibet Plateau and Hengduan Mountains. There are also abundant *Q. preguyavaefolia* Tao (Zhou, 1992) fossils in the late-Cenozoic strata of this region (Zhou, 1999). For example, the Longmen flora (Su *et al*., 2013) and the Fudong flora (Tao, 1986; Huang *et al*., 2013) in south-western China, both from the Sanying Formation of the Pliocene, are dominated by *Q. preguyavaefolia* fossils; the Namling flora in Tibet from the Wulong Formation (middle Miocene, 15 Ma) is the earliest recorded occurrence of *Q. preguyavaefolia* fossils (Li and Guo, 1976; Spicer *et al*., 2003). Together, these fossils provide ideal material to estimate the atmospheric CO_2 concentration history of the late Cenozoic and to study the CO_2–temperature relationship during warm climate intervals in the mid-Miocene climatic optimum and the middle to late Pliocene. In this study, we chose *Q. guyavifolia* (the NLR of *Q. preguyavaefolia* fossils, Fig. 2) in order to (1) determine how the stomatal frequency of *Q. guyavifolia* responds to decreasing pCO_2 (increasing altitude) and to generate calibration curves of stomatal frequency vs. atmospheric pCO_2; (2) test whether samples collected along an altitudinal gradient provide results consistent with results from historical herbarium specimens; (3) estimate the late-Pliocene atmospheric CO_2 concentration using two contemporaneous *Q. preguyavaefolia* fossils; and (4) compare CO_2 levels estimated using the stomatal frequency of *Q. guyavifolia* with previous estimates. This is the first study to use both extant field collections from along an altitudinal gradient and historical herbarium specimens to establish a specific stomatal frequency–pCO_2 relationship.

MATERIALS AND METHODS

Collection of extant altitudinal material, historical herbarium specimens and fossil samples

Extant sun and shade leaves of *Quercus guyavifolia* were collected from five individuals at each of 17 sites at elevations ranging from 2493 to 4497 m (the altitudinal range of *Q. guyavifolia* distributions is approx. 2500–4500 m), i.e. pCO_2 22·695–29·134 Pa (Supplementary Data Table S1; Fig. 1). Sun and shade leaves were collected because light intensity affects these two types of leaves differently and has a positive effect on the stomatal frequency (Kürschner, 1997; Royer, 2001; Lake *et al*., 2002; McElwain, 2004; Kouwenberg *et al*., 2007). Sun leaves were collected from outer branches; shade leaves were collected from within and beneath canopies. To account for the high natural population variability in stomatal frequency (Poole and Kürschner, 1999; Beerling and Royer, 2002*a*), four sun and four shade leaves were collected from each of five *Q. guyavifolia* trees at each site.

Herbarium samples of *Q. guyavifolia* from the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN) were examined and they spanned the time period 1926–1995. Specimens from similar altitudes (range 2800–3300 m) were chosen (Supplementary Data Table S2) so as to limit stomatal frequency variation that might be caused by factors associated with changing altitude. Sample HS006 collected in the field extends the time period to 2011, i.e. pCO_2 21·207–26·539 Pa (Table S2).

Fossil leaves of *Q. preguyavaefolia* (Fig. 2E–H) were collected from two different late-Pliocene floras – the Hunshuitang and the Qingfucun floras (Fig. 1). The Hunshuitang flora collection site is located 25 km north-east of Kunming, central Yunnan Province, south-western China (25°06'N, 102°57'E, altitude 2102 m). The Hunshuitang flora belongs to the Ciying Formation from the late Pliocene (Yunnan Bureau of Geology and Mineral Resources, 1978, 1990; Jiang *et al*., 2003). The Qingfucun flora, located in Weixi county, north-western Yunnan (27°05'N, 99°21'E, altitude 2476 m), is from the Sanying Formation, late Pliocene (3·6 Ma; Li *et al*., 2013). We analysed five fossil specimens of *Q. preguyavaefolia* from the Hunshuitang flora and four from the Qingfucun flora.

Stomatal analysis of extant samples collected along an altitudinal gradient

Mature leaves (1–2 years of leaf growth, mostly 2 years) were chosen for cuticle preparation. Leaf fragments of *Q. guyavifolia* were macerated using a 1:1 solution of glacial acetic acid and 30 % H_2O_2 at 80°C for approx. 3 h after the thick trichomes were removed. The lower cuticles without mesophyll tissue were stained using safranin O solution and then mounted in glycerine on glass slides (Stace, 1965; Poole and Kürschner, 1999). Cuticle images were taken using a light microscope (Leica DM 1000) attached to a Leica DFC 295 camera. The size of the images for stomatal and epidermal cell counts was $468 \times 351\ \mu m^2$ (approx. 0·1643 mm^2). In addition, cleared leaves of *Q. guyavifolia* (Fig. 2C, D) were made following the protocol of Hickey and Wolfe (1975) to compare the leaf morphology of extant and fossil material.

Stomatal density (SD) is measured as the number of stomata per mm^2 of leaf. Stomatal index (SI) is the proportion of stomata to the total number of epidermal cells and measured as:

$$SI(\%) = [SD/(SD + ED)] \times 100 \text{(Salisbury, 1927)} \quad (1)$$

where ED is epidermal cell density.

Stomatal and epidermal cells were counted using the software package ImageJ version 1.42q (http://rsb.info.nih.gov/ij). Samples came from five individuals at each site; four leaves were taken from each individual; three microscope fields were counted per leaf. The leaves of *Q. guyavifolia* are hypostomatous (He *et al*., 1994) so the images of stomatal and epidermal cell counts were all made on the abaxial surface. This resulted in 60 counts (5 individuals × 4 leaves × 3 counts) for each site. Stomatal and epidermal cells were counted in intercostal areas and restricted to the mid-lamina region to minimize variability (Poole *et al*., 1996). The SD and SI for each site were calculated as the mean of 60 counts per site.

Stomatal analysis of historical herbarium material

The experimental protocols for cuticle preparation of herbarium samples were the same as for extant field material. Three mature leaves were collected from each historical herbarium specimen; five microscope fields were counted per leaf, thus 15 counts were made for each specimen.

Stomatal analysis of fossil material

Fossil leaf fragments were treated successively with 20 % HCl, 40 % HF and 20 % HCl again to remove calcareous and siliceous materials, and then macerated using 3·5 % NaClO solution for 10 min to 1 h until they became white or translucent. After removing the mesophyll tissue, the lower cuticles were stained using safranin O and mounted in glycerine on glass slides (Ye, 1981; Kerp, 1990; Leng, 2000). Cuticular images were taken using a light microscope (Leica DM 750) linked to a Leica DFC 295 camera. The size of the images was $298 \times 223\ \mu m^2$ (approx. 0·0665 mm^2). Five to ten cuticular images were counted for each fossil; a separate mean of the counts was calculated for the two fossil sites. The cuticles examined in fossil leaves were from the same part of the leaf as those for the extant leaves (intercostal area near mid-lamina). All cuticular slides were deposited at the Laboratory of Environmental Change and Its Impact on Plants Group in the Kunming Institute of Botany, Chinese Academy of Sciences.

Calibration curves

Calibration curves of stomatal frequency vs. pCO_2 for sun and shade leaves were constructed based on extant samples. Atmospheric pCO_2 used in the calibration curves were calculated from the elevation range using eqn (2) (Beerling and Royer, 2002*a*, derived from Jones, 1992):

$$elev(p_2) = -\ln\left(\frac{p_2}{p_1}\right) \times \frac{R \times T}{(M_A \times g)}, \quad (2)$$

where p_1 and p_2 are the CO_2 partial pressures (Pa) at sea level and at the site, respectively; R is the gas constant

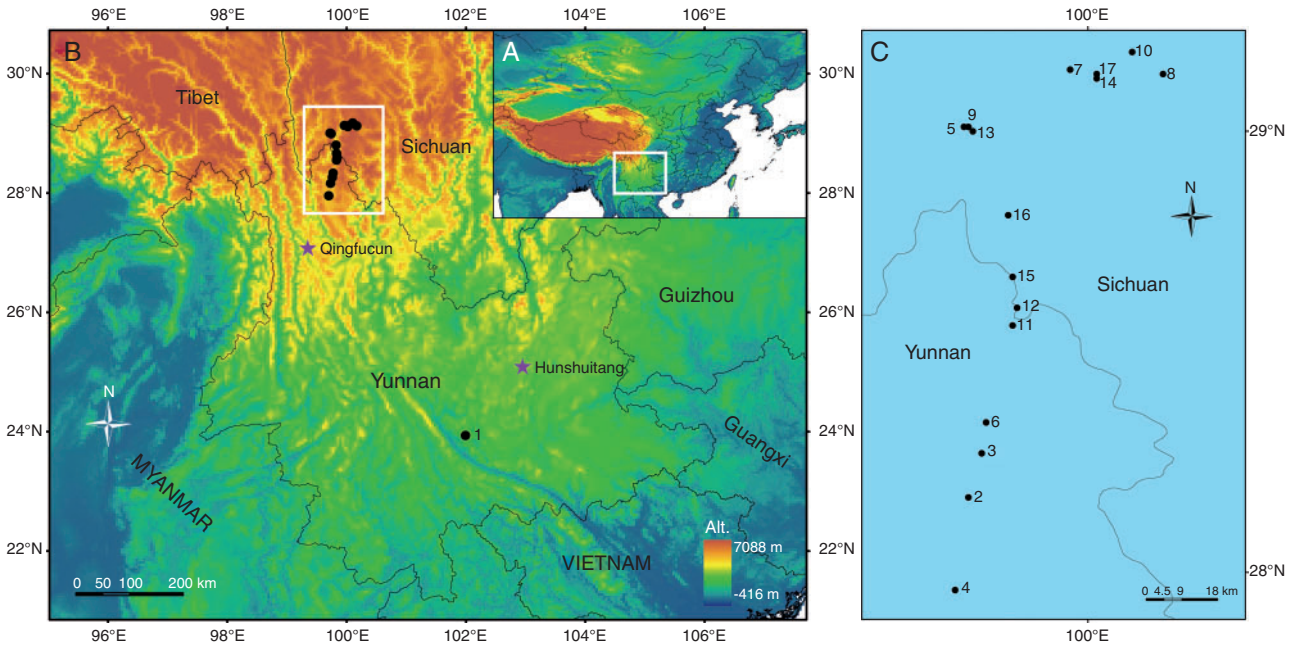

FIG. 1. The locations of 17 sites (black points) where extant field samples of leaf materials of *Quercus guyavifolia* were collected and two sites (purple stars) where fossil materials of *Q. preguyavaefolia* were collected. (A) The study area. (B) Locations where extant and fossil leaf materials were collected. (C) Detailed location map showing 16 of the 17 collection sites in the boundary (grey line) region between Yunnan and Sichuan Provinces.

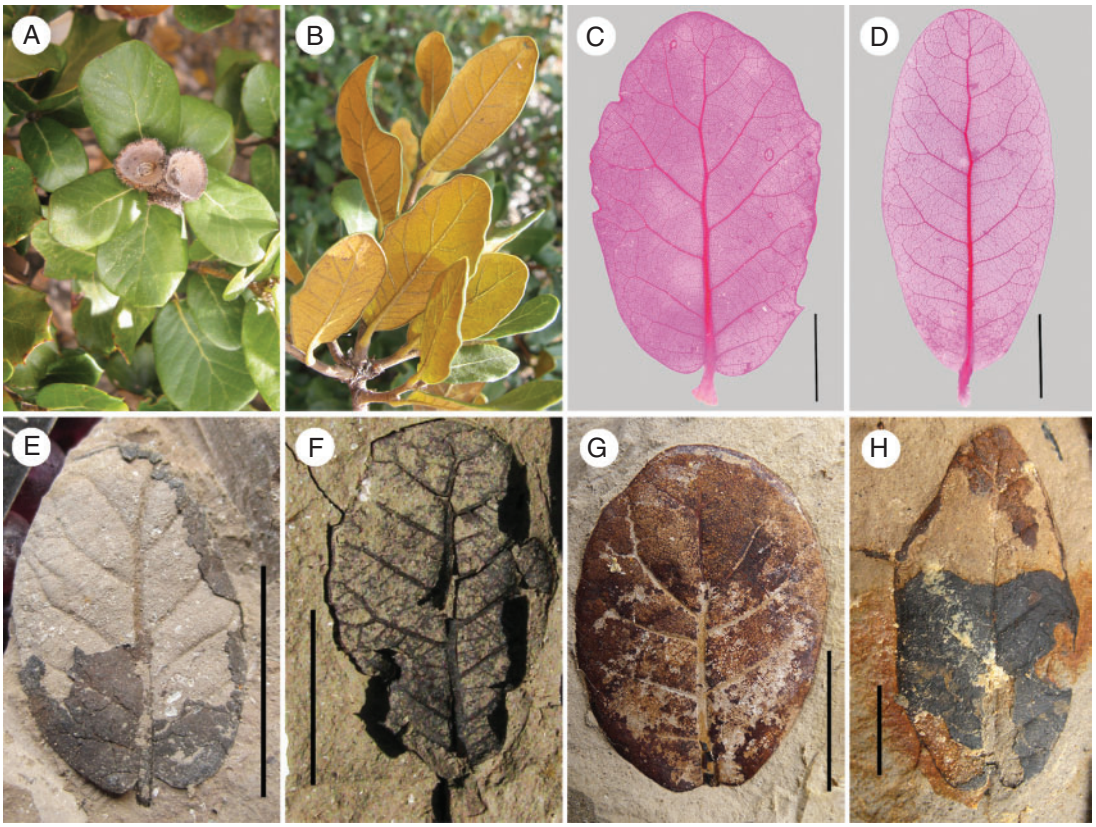

FIG. 2. Comparisons of leaf morphology of extant *Quercus guyavifolia* and fossil *Q. preguyavaefolia*. (A, B) Branches of extant *Q. guyavifolia*. (C, D) Cleared leaves of extant *Q. guyavifolia*. (E, F) and (G, H) are leaf fossils from the Hunshuitang flora and the Qingfucun flora respectively. Scale bars = 1 cm.

($8{\cdot}3144\,Pa\,m^3\,mol^{-1}\,K^{-1}$); T is the mean annual temperature (K) of the range in elevation; M_A is the molecular weight of air ($0{\cdot}028964\,kg\,mol^{-1}$); g is the acceleration due to gravity ($9{\cdot}8\,m\,s^{-2}$); and *elev* (p_2) is the elevation (m) of the site. As altitude increases, pCO_2 decreases from 29·134 to 22·695 Pa (Supplementary Data Table S1). The calculated atmospheric pCO_2 should have no significant difference from the field atmospheric pCO_2 (McElwain, 2004).

Calibration curves were constructed using linear regression analysis, using R version 2.14.1 (http://www.R-project.org). To determine if sun and shade leaves should be analysed separately or combined, differences in slopes and *y*-intercepts of their constructed curves were tested by analysis of covariance using SPSS Statistics version 18.0 (http://www.spss.com.cn).

Calibration curves of stomatal frequency vs. pCO_2 were also constructed using historical herbarium materials. Historical levels of atmospheric CO_2 at sea level were obtained from Etheridge *et al*. (1996) and from the CO_2 Now website (http://co2now.org/). Using this information, atmospheric pCO_2 at the sites was calculated using eqn (2).

Palaeo-CO_2 estimate

The stomatal frequency of the fossil material was analysed and applied to the calibration curves prepared using the extant field materials and historical herbarium samples, respectively, to estimate palaeo-CO_2 levels during the late Pliocene. Envelopes of uncertainty were obtained after propagating uncertainties in the calibration function and fossil leaf measurements. Due to differences between sun and shade leaves, it was necessary to construct morphotype-specific calibration curves for each type of leaf. Sun leaves are characterized by straight to rounded epidermal cell walls (Fig. 3A, B) whereas shade leaves show a pronounced undulation of the epidermal cell walls (Fig. 3C, D) (Kürschner, 1997), and on this basis fossils from the Hunshuitang flora (Figs 2E, F and 3E, F) and the Qingfucun flora (Figs 2G, H and 3G, H) were all assessed to be sun leaves. Sun leaves of historical herbarium samples were also chosen according to their epidermal features. This necessarily determined that the calibration curve for CO_2 estimation was prepared exclusively using sun leaves of *Q. guyavifolia*; the calibration curve generated using shade leaves will be used in future work for *Q. preguyavaefolia* fossils from different sites. We then compared our atmospheric CO_2 concentration estimates from the Hunshuitang and Qingfucun floras with other published results of atmospheric CO_2 concentration during the late Pliocene (van der Burgh *et al*., 1993; Kürschner *et al*., 1996; Pearson and Palmer, 2000; Tripati *et al*., 2009; Seki *et al*., 2010).

RESULTS

Stomatal frequency of extant Quercus guyavifolia *and calibration curves*

The calibration curves show a significant ($P \leq 0{\cdot}01$) positive linear relationship between stomatal frequency and atmospheric pCO_2 for both sun and shade leaves in extant field samples of *Quercus guyavifolia* collected along an altitudinal gradient (Supplementary Data Table S1; Fig. 4).

The slopes of the calibration curves constructed by sun and shade leaves, respectively, are not different ($P > 0{\cdot}05$). The SD and SI of shade leaves were 1·4 and 7·8 %, respectively, lower than those of sun leaves. There was a significant difference in intercept of SI–pCO_2 curves between sun and shade leaves ($P < 0{\cdot}05$), but not for SD–pCO_2 curves ($P > 0{\cdot}05$), indicating that the SI of shade leaves was lower than that of sun leaves, but the SD was not.

Stomatal frequency of historical herbarium materials and calibration curves

There was no significant relationship between SD and atmospheric pCO_2 for historical herbarium materials, but a significant ($P < 0{\cdot}05$) positive linear relationship between SI and pCO_2 was found (Fig. 5).

Palaeo-CO_2 estimate of the late Pliocene

The relationship between SI and atmospheric pCO_2 for both extant specimens collected along an altitudinal gradient and historical herbarium materials were compared (Fig. 6). Results from both sources showed a significant positive linear relationship between SI and pCO_2 (Fig. 6).

Fossils from the Hunshuitang flora had a mean SD of $516 \pm 91\,mm^{-2}$, and a mean SI of $6{\cdot}69 \pm 0{\cdot}76$ % (Table 1). Using the SD and SI of fossil material, the palaeo-CO_2 concentration was estimated using the equations in Fig. 4A and 4C, respectively, which were both derived from extant sun leaves (Table 1). The palaeo-CO_2 concentration was also estimated using the equation in Fig. 5B which was derived from sun leaves of historical specimens (Table 1; Fig. 6).

Fossils from the Qingfucun flora had a mean SD of $496 \pm 24\,mm^{-2}$, and a mean SI of $7{\cdot}79 \pm 0{\cdot}1$ % (Table 1). Palaeo-CO_2 levels of the late Pliocene were estimated by applying the equations in Fig. 4A and 4C to the SD and SI, respectively, of the fossils (Table 1). The palaeo-CO_2 was also estimated using the equation in Fig. 5B to compare with the estimates from the extant field samples (Table 1; Fig. 6).

DISCUSSION

Positive relationship between stomatal frequency and pCO_2 *in* Quercus guyavifolia

We found a positive relationship between stomatal frequency and atmospheric pCO_2 in *Q. guyavifolia* sun and shade leaves collected along an altitudinal gradient, which is consistent with the pattern from the historical herbarium samples. It is an unusual and interesting phenomenon because most other species show an inverse relationship (Woodward, 1987; van der Burgh *et al*., 1993; Woodward and Kelly, 1995; Beerling and Royer, 2002*b*; Royer, 2003; Kouwenberg *et al*., 2007; Franks and Beerling, 2009). However, several studies also report that in some species stomatal frequency increases with atmospheric CO_2 concentration (Ferris and Taylor, 1994; Royer, 2001). In particular, some *Quercus* species such as *Q. rubra* and *Q. robur* grown in climate-controlled greenhouses show an increase in

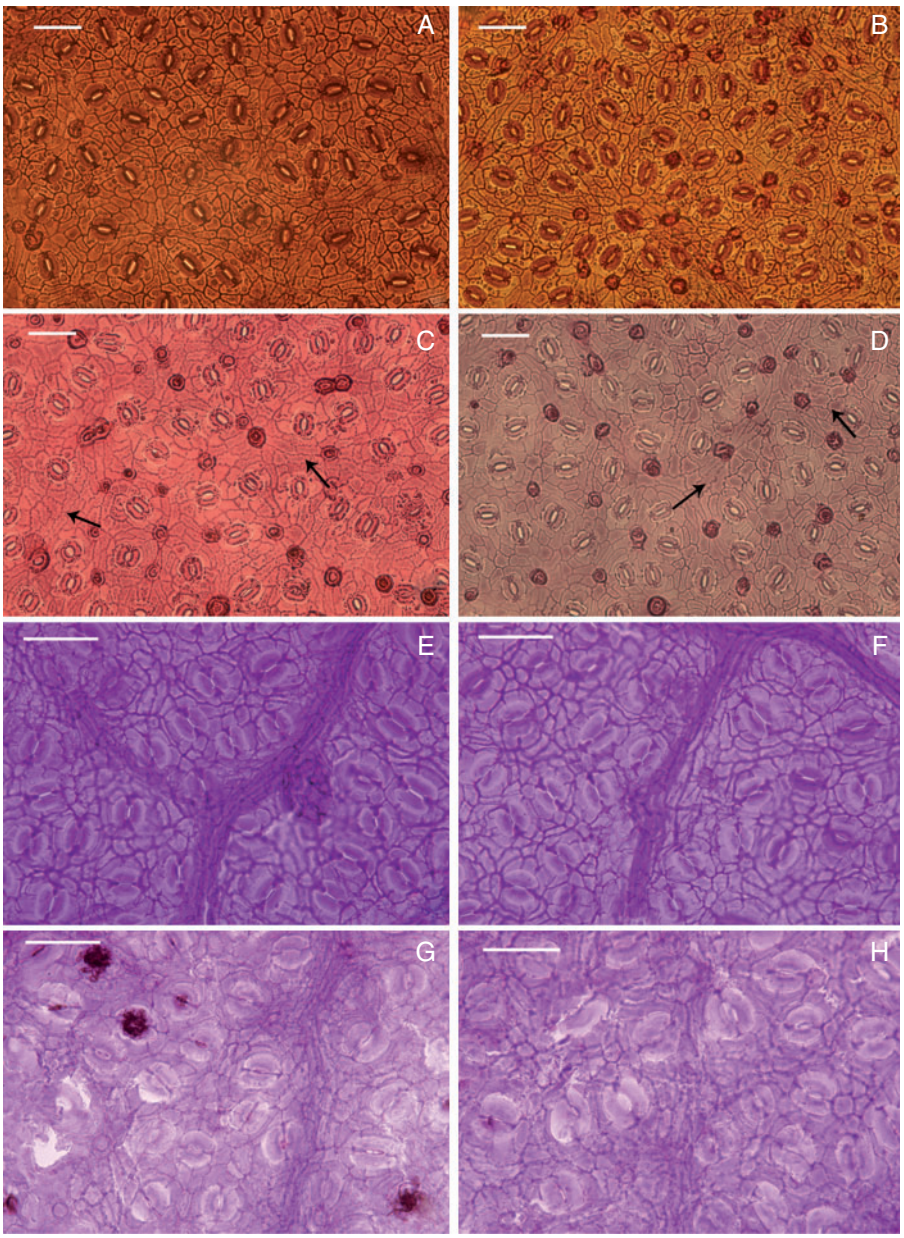

FIG. 3. Images of the cuticle of sun (A, B) and shade (C, D) leaves of extant *Quercus guyavifolia* and *Q. preguyavaefolia* fossils from the Hunshuitang (E, F) and Qingfucun (G, H) floras. Scale bars = 50 μm. Black arrows indicate the undulant epidermal cell walls in shade leaves.

SD at elevated CO_2 (Dixon *et al*., 1995; Atkinson *et al*., 1997). Although greenhouse results are not necessarily reliable, these results indicate to a certain extent that a positive relationship between stomatal frequency and atmospheric pCO_2 in other species of *Quercus* may be not unexpected. Recently, this positive correlation between SI and atmospheric pCO_2 in historical herbarium specimens of *Typha orientalis* has been used to estimate atmospheric CO_2 during the Plio-Pleistocene transition (Bai *et al*., 2014). This is supportive evidence of the reliability of the positive relationship used to estimate palaeo-CO_2.

The positive relationship detected between stomatal frequency and atmospheric pCO_2 in *Q. guyavifolia* was observed from both the altitudinal collection and the historical herbarium samples. These two independently derived results showed the same pattern. The altitudinal samples were collected in Yunnan and Sichuan provinces; the historical samples were collected from similar altitudes in northern and north-western Yunnan province. To test if this unusual, positive relationship between pCO_2 and stomatal frequency is affected by other environmental factors, the relationships between stomatal frequency and other climatic factors (mean annual temperature, mean annual precipitation, annual mean relative humidity) were tested by simple linear regression analysis. The result showed that only atmospheric pCO_2 significantly correlates to stomatal frequency of *Q. guyavifolia* (Figs 4 and 5) rather than the mean

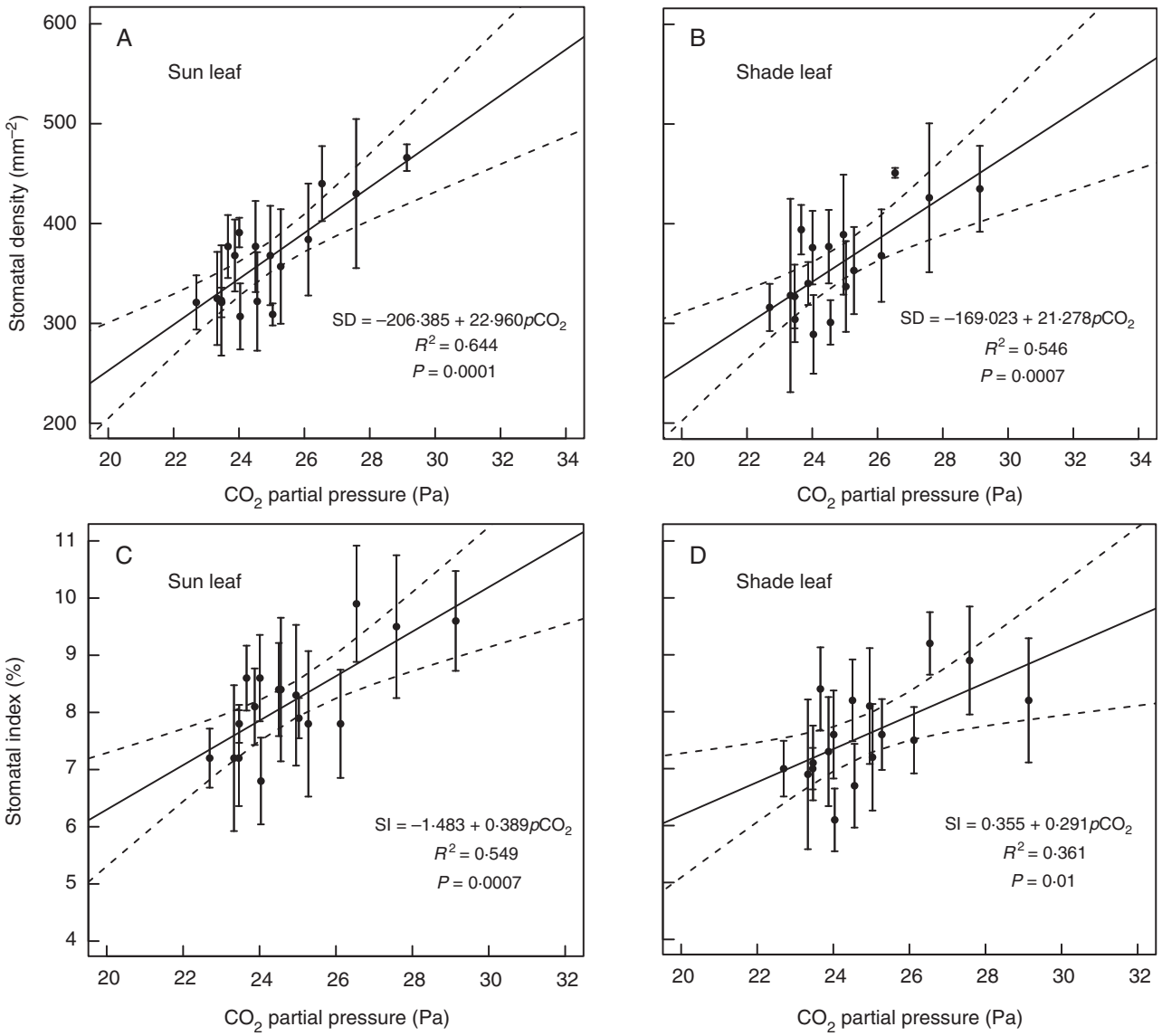

FIG. 4. Relationship between stomatal frequency (A, B, stomatal density; and C, D stomatal index) and CO_2 partial pressure of *Quercus guyavifolia* sun (A, C) and shade (B, D) leaves. Error bars are ±1 s.d. The solid line indicates the best fit in classical regression analysis. Dashed lines are 95 % confidence limits.

annual temperature ($P > 0{\cdot}05$ for both the altitudinal collection and the historical herbarium samples), mean annual precipitation ($P > 0{\cdot}05$ for both the altitudinal collection and the historical herbarium samples) and annual mean relative humidity ($P > 0{\cdot}05$ for both the altitudinal collection and the historical herbarium samples), confirming that the positive relationship between stomatal frequency and pCO_2 in *Q. guyavifolia* is determined by atmospheric pCO_2. Together with evidence from other studies (Dixon *et al.*, 1995; Atkinson *et al.*, 1997; Bai *et al.*, 2014), we propose that the positive relationship between stomatal frequency and pCO_2 in *Q. guyavifolia* is reliable and this relationship can be used as a basis to estimate palaeo-CO_2 levels.

The physiological mechanism underlying the positive stomatal response to pCO_2 is probably complicated. Stomata play a central role in the uptake of photosynthetic CO_2 and water loss from the leaf. Both physiological (stomatal aperture change) and morphological (SD change) strategies can be used by plants to regulate gas exchange (Haworth *et al.*, 2013). The positive stomatal response to pCO_2 may be contributed by multiple factors. One of the factors is possibly leaf nitrogen content. Previous studies found that elevated CO_2 concentration can increase leaf nitrogen content in jack pine and white birch seedlings (Zhang and Dang, 2005). Plants of *Q. pannosa* (= *Q. guyavifolia*) and *Q. aquifolioides* grown at lower altitudes (higher atmospheric pCO_2) have higher leaf nitrogen content (or higher nitrogen allocation in the photosynthetic system), stomatal conductance, photosynthetic rate and carboxylation rate (Zhang *et al.*, 2005; Feng *et al.*, 2013). High leaf nitrogen content significantly increases carboxylation capacity (Rogers *et al.*, 1998; Pérez *et al.*, 2011) and consequently results in a decrease in the ratio of the intercellular to atmospheric CO_2

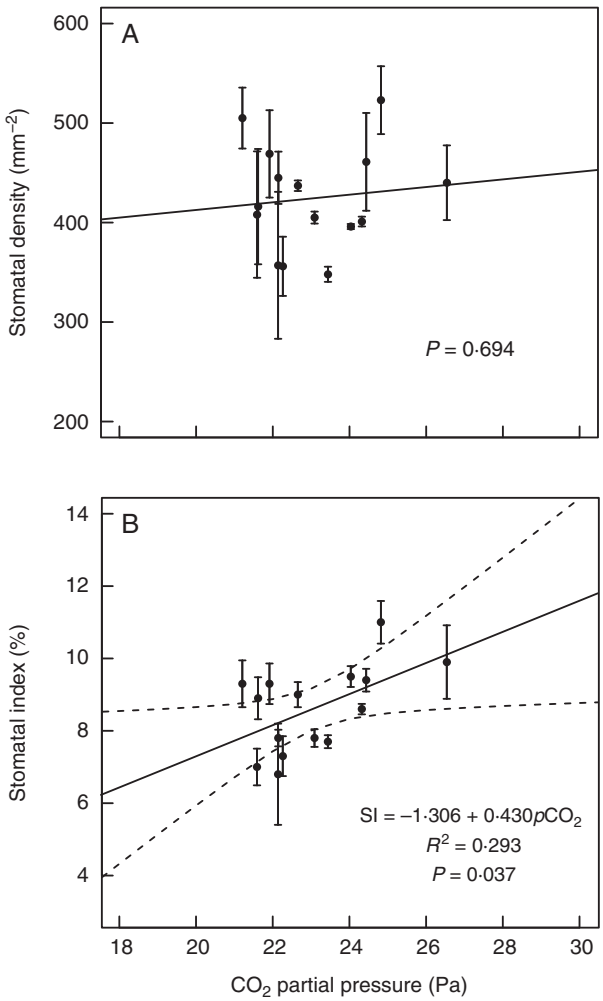

FIG. 5. Relationship between stomatal frequency (A, stomatal density and B, stomatal index) and CO_2 partial pressure of *Quercus guyavifolia* historical herbarium specimens. Error bars are ±1 s.d. The solid line indicates the best fit in classical regression analysis. Dashed lines are 95 % confidence limits.

concentration and an increase in $\delta^{13}C$ values (Sasakawa *et al.*, 1989; Cordell *et al.*, 1999), and therefore an increase of SD (Qiang *et al.*, 2003).

Differences in stomatal frequency between sun and shade leaves

Our results confirm that distinguishing sun leaves from shade leaves is necessary when using the stomatal method to estimate palaeo-CO_2 levels (Kürschner, 1997). Stomatal frequency of both sun and shade leaves in *Q. guyavifolia* decreased with decreasing pCO_2. However, SI of shade leaves was lower than that of sun leaves although the SD was not different between sun and shade leaves. This corroborates results from previous studies which showed that stomatal frequency of sun leaves is higher than that of shade leaves (Kürschner, 1997; Wagner, 1998; Royer, 2001; Kouwenberg *et al.*, 2007) resulting from the positive effect of light intensity on stomatal frequency (Lake *et al.*, 2001, 2002; Kouwenberg *et al.*, 2007). Kürschner (1997) reported that the SD of *Q. petraea* sun leaves was about 45 % higher than that of shade leaves, and up to 60 % higher in

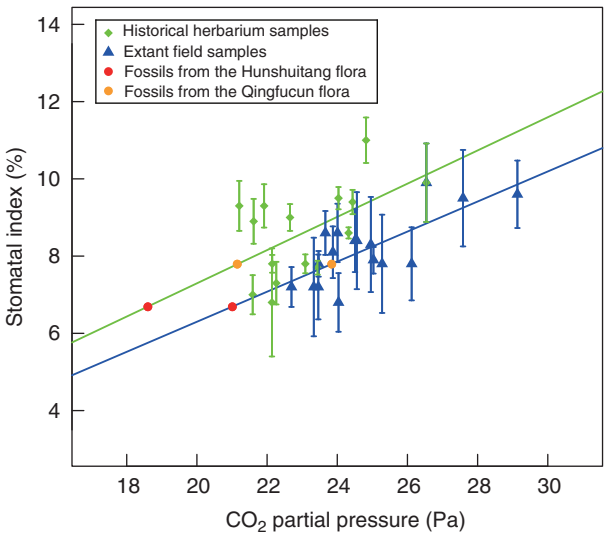

FIG. 6. Correlation curves constructed using the SI of extant field collections along an altitudinal gradient and historical herbarium samples (see key), and comparison of estimated palaeo-pCO_2 values (fossils from the Hunshuitang and Qingfucun flora, as indicated).

fossil leaves of *Q. pseudocastanea*. Kouwenberg *et al.* (2007) also observed a higher SD and SI in sun leaves compared with shade leaves in both *Q. kelloggii* (up to 38 % higher) and *Nothofagus solandri*.

Comparison of stomatal density and stomatal index

The SD and SI gave different estimates for palaeo-CO_2. The SI is a more precise proxy for palaeo-CO_2 estimation than SD because SI removes the effect of other environmental factors such as temperature, water stress and humidity on the size and/or spacing of epidermal cells, which will result in higher or lower SDs (Salisbury, 1927; Kürschner *et al.*, 1996; Kürschner, 1997; Royer, 2001; Sun *et al.*, 2003; McElwain, 2005; Kouwenberg *et al.*, 2007; Haworth *et al.*, 2010*a*). Here we showed that the SI of historical herbarium samples had a significant positive response to atmospheric pCO_2 but SD did not, confirming that SD is more variable than SI and therefore not as reliable as SI for palaeo-pCO_2 estimation. However, in practice, many fossil leaves are not well preserved and epidermal cells are difficult to identify. Thus SI analysis is impossible and SD becomes the sole option but may give rise to error. Fortunately, our fossils were well preserved and SI could be accurately calculated. Therefore, our results are probably more precise than those derived only by SD. We also used SD for palaeo-pCO_2 calculation in order to compare with the results derived from SI (Table 1). Our comparison of palaeo-pCO_2 levels estimated from SD and SI provides an example of overpredicting pCO_2 levels using SD.

Late-Pliocene atmospheric CO_2 levels

As a test of the applicability and reliability of the positive relationship of stomatal frequency and pCO_2 in *Q. guyavifolia* as

TABLE 1. *Estimates of CO_2 partial pressure (pCO_2) derived by using the calibration curves of stomatal frequency and* pCO_2 *in* Quercus guyavifolia *sun leaves from both extant field samples (Fig. 4A, C) and historical herbarium samples (Fig. 5B)*

Sample	Age	No. of fossils	Total image counts	SF (mean ± s.d.)	pCO_2-E (mean ± s.e.)	pCO_2-H (mean ± s.e.)
Hunshuitang flora	Late Pliocene	5	46	SD: 516 ± 91 mm^{-2}	31·46 ± 2·79 Pa (310·51 ± 27·51 ppm)	–
				SI: 6·69 ± 0·76 %	21·01 ± 3·47 Pa (207·36 ± 34·25 ppm)	18·60 ± 5·53 Pa (183·52 ± 54·63 ppm)
Qingfucun flora	Late Pliocene, 3·6 Ma	4	34	SD: 496 ± 24 mm^{-2}	30·59 ± 1·57 Pa (301·92 ± 15·47 ppm)	–
				SI: 7·79 ± 0·1 %	23·84 ± 2·57 Pa (235·26 ± 25·37 ppm)	21·15 ± 3·84 Pa (208·77 ± 37·87 ppm)

SF, stomatal frequency; pCO_2-E, estimates of CO_2 partial pressure from extant field samples; pCO_2-H, estimates of CO_2 partial pressure from historical herbarium samples.

a proxy for palaeo-pCO_2 levels, palaeo-pCO_2 was estimated using *Q. preguyavaefolia* fossils. Our results derived using the SI calibration data set indicate late-Pliocene atmospheric CO_2 levels of approx. 210 and 240 ppm derived using extant field collections and approx. 180 and 210 ppm derived from herbarium samples, which are consistent with most other estimates. Beerling and Royer (2011) compiled 370 estimates of Cenozoic atmospheric CO_2 levels obtained using different protocols; they show that since the Miocene most atmospheric CO_2 estimates were lower than present-day levels of approx. 390 ppm. We compared our palaeo-CO_2 estimates during the late Pliocene with those based on stomatal frequency of other species (terrestrial-based proxy) and other proxies such as alkenone and boron (marine-based proxies) for the same time period (Fig. 7). All of the estimates indicate palaeo-CO_2 levels between approx. 190 and approx. 430 ppm for the late Pliocene, with the majority indicating that atmospheric CO_2 levels in the late Pliocene were lower than at present. However, even atmospheric CO_2 estimates derived from the same source (terrestrial-based or marine-based proxies) are still quite different. Therefore, atmospheric CO_2 estimates to date have not been sufficient to account for the warm climate interval during the late Pliocene. More research is required to obtain more precise estimates of palaeo-CO_2 during this period.

In addition, our comparatively low atmospheric CO_2 values may be related to elevations in the fossil sites. It is generally accepted that the Qinghai-Tibet Plateau reached its current height in the late Miocene and retained it (Spicer *et al.*, 2003); previous studies have also showed that western Yunnan had approached its highest altitude before the late Pliocene (Sun *et al.*, 2011). The palaeoaltitude of the Xianfeng flora, about 60 km away from the Hunshuitang locality, was about 1936 m in the late Miocene (Jacques *et al.*, 2014). This means that the altitudes of our fossil sites in the late Pliocene were probably similar to present-day altitudes (approx. 2000–2500 m). If the palaeoaltitudes of the two sites are taken into account, atmospheric CO_2 levels during the late Pliocene should be approx. 270 and 320 ppm derived by extant field collections and approx. 240 and 280 ppm derived by herbarium samples. Thus, these results are very similar and correspond well to other estimates, confirming that the positive relationship between stomatal frequency and atmospheric pCO_2 in *Q. guyavifolia* is reliable as a proxy for estimating palaeo-CO_2 levels.

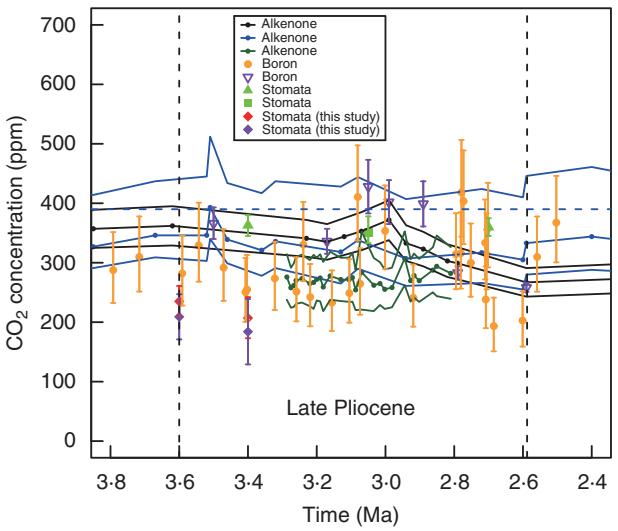

FIG. 7. Estimates of palaeo-CO_2 concentration during the late Pliocene derived using different methods. The palaeo-CO_2 estimates derived from alkenone (see key; black lines above and below represent uncertainties) and boron (purple) are cited from Seki *et al.* (2010) modified by Beerling and Royer (2011); alkenone (blue lines above and below represent uncertainties; Zhang *et al.*, 2013); alkenone (dark green lines above and below represent uncertainties; Badger *et al.*, 2013); boron data (orange; Bartoli *et al.*, 2011); stomata data (van der Burgh *et al.*, 1993; Kürschner *et al.*, 1996); stomata data (Stults *et al.*, 2011); stomatal data (red) and stomatal data (purple) are palaeo-CO_2 levels estimated from field collections along an altitudinal gradient and from historical herbarium samples respectively (this study). Error bars represent uncertainties (see Materials and Methods). One of our fossils is 3·6 Ma, and the other is unknown (sometime during the late Pliocene). The period between the two vertical dashed lines is the late Pliocene (3·6–2·588 Ma) (International Chronostratigraphic Chart; version 2013). The blue horizontal dashed line indicates recent levels of atmospheric CO_2 concentration (390 ppm).

Actually, a correction for pCO_2 at the elevation is necessary to obtain more accurate results. Clearly, when attempting to reconstruct palaeo-pCO_2 using the stomatal frequency of fossils, the pCO_2 estimates will be influenced by the elevation at which the now-fossilized plants were growing. Thus, to obtain more

precise pCO_2 estimates it is essential to apply a correction factor for pCO_2 related to altitude. This should also be done for samples collected along an altitudinal range (McElwain, 2004) and for historical herbarium samples (Greenwood *et al*., 2003) when constructing the stomatal frequency–pCO_2 curve. Unfortunately, few previous studies have made this correction on either extant specimens, herbarium specimens or fossils. Therefore, we suggest that future studies should incorporate a correction factor related to altitude for all specimens and samples.

Recently, there has been much debate about whether the linear relationship between stomatal frequency and pCO_2 continues when the ambient CO_2 concentration rises beyond approx. 500 ppm, because the stomatal frequency of some plant species may lose sensitivity at these high levels (Woodward and Bazzaz, 1988; Kürschner *et al*., 1997; Beerling and Royer, 2002*a*, *b*; Beerling *et al*., 2009; Haworth *et al*., 2011*a*). Nevertheless, the pCO_2 estimate reported in this study, and the stomatal frequency–pCO_2 training sets are applicable because atmospheric CO_2 levels during the Pliocene remained below 500 ppm according to our results and those of previous estimates (Fig. 7).

Conclusions

We have shown a significant positive relationship between atmospheric pCO_2 and stomatal frequency in *Q. guyavifolia* that can be used as a proxy to estimate late-Cenozoic palaeo-CO_2 concentrations. This is the first study in which both field samples collected along an altitudinal gradient and historical herbarium samples of a single species have been used to estimate palaeo-CO_2 concentration. In addition, a positive relationship between stomatal frequency and pCO_2 has seldom been used to estimate palaeo-CO_2 concentration. Our estimated palaeo-CO_2 concentration provides new independent data for late-Cenozoic CO_2 estimates derived using vascular land plants. There are, however, three sources of variation. First, sun and shade leaves give different results of stomatal frequency, confirming that it is necessary to analyse sun and shade leaves separately when using the stomatal method to estimate palaeo-CO_2. Secondly, the estimates derived from SD and SI using sun leaves were also different, so more precise estimates will be possible when we find and analyse fossils that have well-preserved shade leaves in addition to sun leaves. Thirdly, our research concluded that atmospheric CO_2 levels in the late Pliocene were approx. 180–240 ppm. Although these results are consistent with other studies reporting CO_2 levels lower than modern atmospheric concentrations, when more appropriate fossil material becomes available then much more accurate estimates will be possible.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: location, altitude, pCO_2, stomatal density and stomatal index of *Q. guyavifolia* sun and shade leaves where extant field samples were collected. Table S2: collection time, location, altitude, pCO_2, stomatal density and stomatal index of *Q. guyavifolia* sun leaves from historical herbarium specimens.

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