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ASSESSING THE POTENTIAL FOR THE STOMATAL CHARACTERS OF EXTANT AND FOSSIL *GINKGO* LEAVES TO SIGNAL ATMOSPHERIC CO_2 CHANGE[1]

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The stomatal density and index of fossil *Ginkgo* leaves (Early Jurassic to Early Cretaceous) have been investigated to test whether these plant fossils provide evidence for CO_2-rich atmosphere in the Mesozoic. We first assessed five sources of natural variation in the stomatal density and index of extant *Gingko biloba* leaves: (1) timing of leaf maturation, (2) young vs. fully developed leaves, (3) short shoots vs. long shoots, (4) position in the canopy, and (5) male vs. female trees. Our analysis indicated that some significant differences in leaf stomatal density and index were evident arising from these considerations. However, this variability was considerably less than the difference in leaf stomatal density and index between modern and fossil samples, with the stomatal index of four species of Mesozoic *Ginkgo* (*G. coriacea, G. huttoni, G. yimaensis,* and *G. obrutschewii*) 60–40% lower than the modern values recorded in this study for extant *G. biloba.* Calculated as stomatal ratios (the stomatal index of the fossil leaves relative to the modern value), the values generally tracked the CO_2 variations predicted by a long-term carbon cycle model confirming the utility of this plant group to provide a reasonable measure of ancient atmospheric CO_2 change.

Key words: atmospheric CO_2; cuticles; *Ginkgo*; plant fossils; stomatal density; stomatal index; stomatal ratio.

Woodward (1987) investigated the stomatal density of eight species of forest trees by observations on herbarium records covering a 200-yr period and showed a 40% reduction in relation to a rise in atmospheric CO_2 of 60 μmol/mol over that time. Peñuelas and Matamala (1990) studied the stomatal density of 14 species of herbarium material collected 240 yr ago, arriving at a similar conclusion to that of Woodward (1987). These observations were reproduced by exposing some of the same tree species to reduced CO_2 concentrations in controlled environment experiments (Woodward, 1987; Woodward and Bazzaz, 1988). Moreover, these experiments showed that the CO_2 levels to which leaves were exposed during growth induced changes in their stomatal index (proportion of stomata to epidermal cells). The changes in turn affected stomatal conductance and WUE (water using efficiency) (Woodward and Bazzaz, 1988; Berryman, Eamus, and Duff, 1994; Beerling, McElwain, and Osborne, 1998).

Following these results, studies on Late Quaternary plant fossils indicate a similar general inverse relationship between stomatal density and atmospheric CO_2 concentration (Beerling and Chaloner, 1992, 1993b; Beerling, 1993; Paoletti and Gellini, 1993; Van der Water, Leavitt, and Betancourt, 1994; McElwain, Mitchell, and Jones, 1995; Kürschner, 1996; Kürschner et al., 1996; Wagner, 1998). For the Late Neogene, Van der Burgh et al. (1993) used this relationship to make quantitative estimates of atmospheric CO_2 concentration from the stomatal indices of fossil *Quercus petraea* leaves.

As extant species of vascular plants are generally not known in the fossil record earlier than the Late Tertiary, McElwain and Chaloner (1995) suggested the concept and method of using nearest living equivalent (NLE) species for comparison of living and fossil stomatal parameters. These are defined as species from the present day that are of comparable ecological setting and/or structural similarity to their fossil counterparts and, as far as possible, of close affinity. Using this method, they attempted to deduce CO_2 levels from stomatal density and index during Mesozoic and Paleozoic time, including Early Devonian, Late Carboniferous, Early Permian, Middle Jurassic, and Middle Eocene (McElwain and Chaloner, 1995, 1996; McElwain, 1998).

The only available NLE for fossil Ginkgoaceae is the dioecious species *Ginkgo biloba* (McElwain and Chaloner, 1995, 1996; Beerling, McElwain, and Osborne, 1998; McElwain, Beerling, and Woodward, 1999). *Ginkgo* is an ideal taxon for this work because the foliage has a high fossilization potential, owing to its thick cuticle and deciduous habit. Furthermore, leaves of *Ginkgo* are abundant and range from the late Triassic to the present day, an interval of at least 200 million years. Moreover, CO_2 enrichment experiments have shown that the stomatal characters of extant *Ginkgo biloba* leaves are sensitive to changes in atmospheric CO_2 concentration (Beerling, McElwain, and Osborne, 1998). No work has yet examined the recent historical response of stomatal density and index of *G. biloba* and so we studied herbarium material collected in the early 1900s, when the CO_2 concentration was ~55 μmol/mol lower than today (Friedli et al., 1986).

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It is important to determine whether factors other than CO_2 concentration influence the stomatal characters (density and index) of *Ginkgo* foliage. Here we consider five sources of possible natural variation of leaves within and between *G. biloba* trees from the Botanical Garden in Beijing, China: (1) timing of leaf maturation (May–November), (2) young vs. fully developed leaves, (3) short (determinate) shoots vs. long (indeterminate) shoots, (4) position in the canopy, and (5) male vs. female trees.

In this work, we also test the earlier suggestion that the stomatal density and index of Mesozoic *Ginkgo* leaves were both lower relative to modern values due to growth in a postulated high-CO_2 environment (Beerling, McElwain, and Osborne, 1998). The Jurassic material from China provides an independent test of previous results on British Middle Jurassic leaves, while the Lower Cretaceous fossils extend the temporal range of samples to test the duration of the high-CO_2 Mesozoic ''greenhouse'' climate (Berner, 1994, 1998).

MATERIALS AND METHODS

Fossil Ginkgo—*Ginkgo coriacea* Florin was collected from the Huolinhe Formation of Lower Cretaceous in Huolinhe, northeastern Inner Mongolia, China (Sun, 1993).

Ginkgo huttoni (Sternberg) Heer was from the Scalby Formation of Middle Jurassic, Scalby Ness, Yorkshire, UK (Harris, Millington, and Miller, 1974).

Ginkgo yimaensis Zhou was from the Yima Formation of Middle Jurassic in Henan Province, China (Zhou and Zhang, 1989).

Ginkgo obrutschewii was from the Dzungaria Formation of Early Jurassic in Xinjiang Province, China (Seward, 1911).

Modern Ginkgo—In 1998, fresh leaves of *G. biloba* (maidenhair tree) were collected from trees ~46 yr old in the botanical garden, Institute of Botany, Chinese Academy of Science, Beijing (PE). The leaves for comparison of male and female trees were collected in 1999 in the same garden. With each sampling, only large leaves at each collecting time were selected for measurement. But leaves of all sizes were collected when the relationship between leaf area and stomatal density and index was investigated. Herbarium specimens of *G. biloba* were from the herbarium in the same institute (PE) originally collected from Shanxi Province, China by Harry Smith in 1924 (specimen number 5538).

Cuticle preparation—A small piece (0.5–1 cm^2) of modern material was taken from the middle part of a leaf and processed for several hours in a 10% CrO_3 solution (chromic acid) to remove the mesophyll layer. This material was then rinsed in water. The leaf fragments were subsequently separated into abaxial and adaxial cuticle and the remains of mesophyll and vascular tissue removed with forceps. The fossil material was macerated in a Schulze's solution (Kerp, 1990) for 30 min to several hours according to the material. All cuticle samples were mounted either in dilute glycerin or glycerine jelly and sealed with Canada balsam.

Replica preparation—In order not to damage the herbarium specimens, we made replicas of all material used, prepared using clear nail polish applied to the abaxial leaf surface. When dry, the film of polish was pulled from the leaf and mounted in water for examination by transmitted light microscopy. The outlines of epidermal cells were obscure using the replica technique; however, the stomata were clearly visible and easily counted (see Fig. 1).

Storage of samples—All cuticle preparations, leaf samples, and fossil samples are stored in the National Museum of Plant History, Beijing, China.

Stomatal counts—Computer-aided determination of stomatal density and index were performed on a Leica Q500IW Image Analysis System. When the image of the epidermis cuticle was on the screen each cell (as judged by the observer) was scored for the count. This means that there was a subjective element in the recognition of each cell counted. Stomatal density and index of extant materials are mean values for 25 (field area 0.231 mm^2 at 20 × 10 × 0.32 magnification) or 50 (field area 0.121 mm^2) counted images from five leaves per sample. Counts were made only within the stomatal bands (intervein bands) for both modern and fossil leaves.

Stomatal density is defined as the number of stomata per square millimeter of leaf surface.

Stomatal index was calculated using the equation of Salisbury (1927) [stomatal index = number of stomata × 100/(number of stomata + number of epidermal cells)].

Stomatal ratio is a ratio of the stomatal index of the NLE species divided by that of the fossil species (Chaloner and McElwain, 1997).

Statistics—All of the data were analyzed using analysis of variance (ANOVA), after first checking for homogeneity of variance and additivity (Sokal and Rohlf, 1981). Transformation was not required in any set of data. For the statistical comparisons we report the F value together with the degrees of freedom of the main effect and the residual, respectively, and its significance.

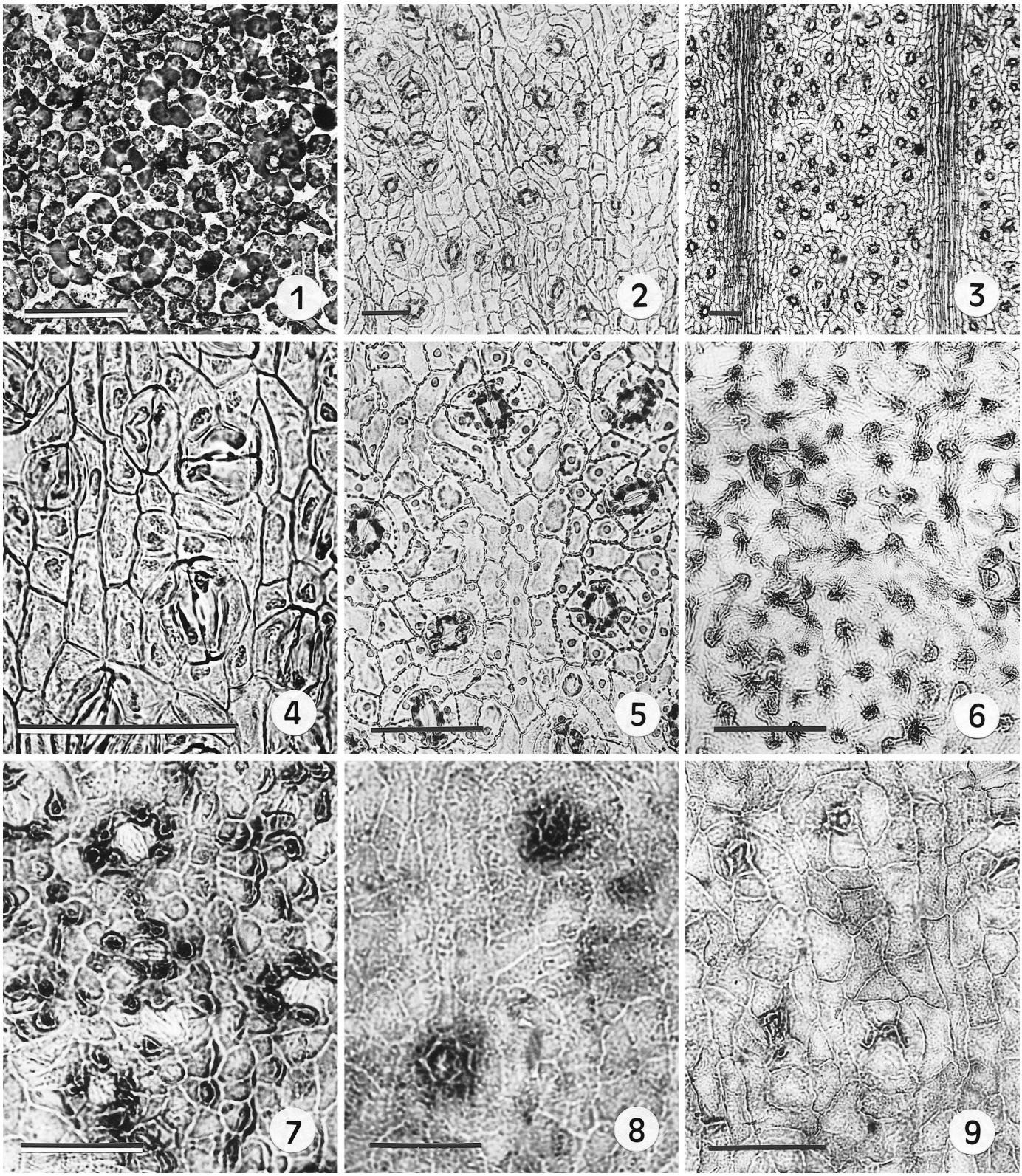
RESULTS AND DISCUSSION

The seasonal variation of leaf stomatal characters—To establish whether leaves maturing at different months in the growing season show differences in stomatal density and index, both were measured on leaves from short shoots that had formed through the year (May to November) (see Fig. 10). ANOVA on these data indicated that timing of maturation in *G. biloba* leaves had no significant effect on stomatal density ($F_{7,54} = 0.41$, $P > 0.05$), but a significant effect on stomatal index ($F_{7,64} = 2.49$, $P = 0.05$). This suggests that, unlike *Quercus robur* (Beerling and Chaloner, 1993a), only the stomatal density of *G. biloba* leaves was insensitive to changes in temperature of ~12°C, as estimated from the long-term climatic data for Beijing between May and November (Müller, 1982).

Leaf stomatal characters of short shoots and long shoots—As in many conifers, the long and short shoots of *G. biloba* are readily distinguished. The leaves are borne spirally on both types, but are widely spaced on the long shoots, while the leaves are crowded, with very short internodes on the short shoot, producing a rosette-like cluster. Leaf buds and leaves of long shoots form and develop in the same growing season, while leaf buds of short shoots form in one growing season, but sprout and develop in the following spring. There is also a morphological distinction between leaves of long shoots and short shoots, with leaves of long shoots being generally bilobate to four-lobed, while those of short shoots are nearly entire to bilobate. Leaves from the female tree were used investigate the differences in leaf stomatal density and index between long and short shoots in June and July (Table 1).

Two-way ANOVA on the stomatal density and index measurements of leaves from long and short shoots indicated that there was a significant difference in stomatal density ($F_{1,32} = 66.45$, $P = 0.001$) but not stomatal index ($F_{1,32} = 0.47$, $P > 0.05$) (Table 1) between shoot types. No significant differences were detected in stomatal density ($F_{1,32} = 0.83$, $P > 0.05$) and index ($F_{1,32} = 0.38$, $P > 0.05$) between the sampling dates for this material (June and July) or for the interaction between date and shoot type (Table 1).

The effect of canopy position on leaf stomatal characters—Leaves of *G. biloba* were collected (September, 1998) from three layers within the canopy (7.0–7.5 m from



Figs. 1–9. The lower epidermal characters of extant and fossil *Ginkgo* leaves. **1.** Replica of lower epidermis on *G. biloba* leaf collected in 1924. Figs. 2–5. *Ginkgo biloba* (collected in 1998). **2.** No significant differentiation between stomata zone and vein zone on a young leaf. **3.** Significant differentiation between stomata zone and vein zone on a developed leaf. **4.** Developing stomata on a young leaf. **5.** Lower leaf epidermis on a developed leaf. Figs. 6–9. Fossil *Ginkgo*. **6.** Lower leaf epidermis of *G. coriacea*. **7.** Lower leaf epidermis of *G. huttoni*. **8.** Lower leaf epidermis of *G. yimaensis*. **9.** Lower leaf epidermis of *G. obrutschewii*. All scale bars = 100 μm.

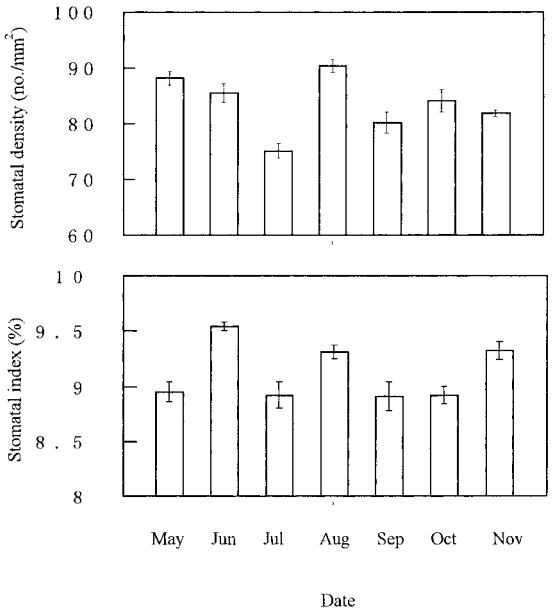


Fig. 10. (Top) Stomatal densities (mean ± 1 SE) and (Bottom) stomatal indices (mean ± 1 SE) of *Ginkgo biloba* leaves collected on a monthly basis in 1998.

the ground surface, 4.5–5.0 m, and 2–2.5 m) on a female tree with a height of 7.8 m; the depth of canopy was 5.8 m (7.8–2 m above the ground). Mature leaves were collected from each height, all from short shoots. ANOVA indicated that there was no significant effect of height on stomatal density ($F_{2,57} = 0.22$, $P > 0.05$), but a significant effect on stomatal index ($F_{2,57} = 5.52$, $P = 0.01$) (see Fig. 11). The female tree sampled was growing in a closely spaced line of trees, and so this ''sun vs. shade'' difference is probably only an approximation of a natural closed woodland. We note that the effect of height, and therefore irradiance, on stomatal density in our *G. biloba* canopy was considerably less than that seen between sun and shade leaves of *Alnus glutinosa* (Poole et al., 1996).

The relationship between leaf area and stomatal characters—Salisbury (1927) noted the negative correlation between leaf area and stomatal density within a species. Stomatal index, however, is thought to be relatively constant with respect to leaf area. Once differentiation of the guard cells has taken place, the ratio of guard cells to epidermal cells will of course be unaltered by leaf expansion, while the absolute stomatal density will fall. Stomatal density is therefore sensitive

TABLE 1. Leaf stomatal density and index of long shoots and short shoots.

	Stomatal density				Stomatal index			
	June		July		June		July	
Shoot type	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Long shoots	124.9	0.7	130.1	0.6	10.1	0.05	9.7	0.08
Short shoots	107.7	0.5	112.9	0.6	9.3	0.03	9.6	0.04

Note: Values are means of five counts on each of five leaves per shoots.

to the state of maturation of the leaf. Stomatal index is remarkably constant, once recognizable stomata can be discerned in the epidermis, i.e., index is independent of any subsequent expansion of the leaf. However, the lack of distinction between veinal and interveinal areas in the smallest immature leaves (<2 cm² area) means that the stomatal index of such juvenile leaves is unreliable.

Based on 12 leaves from long shoots and 19 leaves from short shoots of differing leaf areas, the relationship between leaf area and stomatal density and index was investigated. The results show a general inverse relationship between stomatal density and leaf area with the smallest (i.e., youngest) leaves sampled possessing the highest stomatal densities (Fig. 12). A similar effect is seen for the relationship between stomatal index and leaf area (Fig. 12). However, this arises because the epidermal characters of young *G. biloba* leaves are such that there is no significant differentiation between vein and stomatal bands (Fig. 2); the encircling cells around stomata are not fully developed, but the young stomata can still be observed on the younger leaves (Fig. 4). Since it is easy to differentiate the vein zone and stomatal zone (Fig. 3) in the larger (more mature) leaves, stomatal index is then stable with increasing leaf area. For example, there was no significant correlation between stomatal index and leaf area for the last ten leaves in ascending size sequence (Fig. 12).

Leaf stomatal characters of male and female trees—*Ginkgo biloba* is dioecious, therefore, we next investigated the possibility that the leaf stomatal characters differed on short shoots between female and male trees (N = five trees for each sex).

The results showed that female *G. biloba* trees possessed higher stomatal density than the male trees ($P < 0.05$) (Table 2). In contrast to this result, Beerling et al. (1992) found that female plants of *Salix herbacea* from 2200 m above sea level had significantly ($P < 0.05$) lower abaxial stomatal densities than male plants from the same locality. However, no signifi-

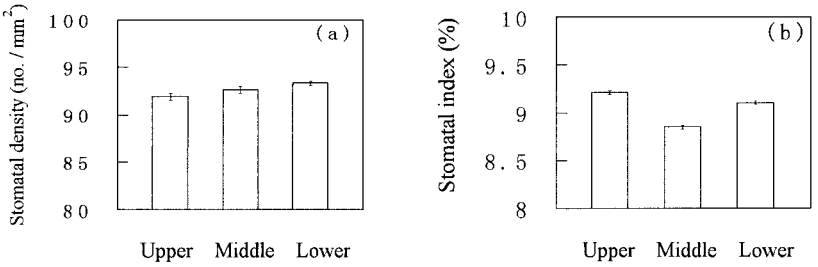


Fig. 11. (a) Stomatal densities (mean ± 1 SE) and (b) stomatal indices (mean ± 1 SE) of *Ginkgo biloba* leaves from different location within the same canopy. Values are means of ten counts per leaf and 20 leaves per canopy layer. Lower, middle, and upper positions correspond to heights in the canopy of 2–2.5, 4.5–5.0, and 7.0–7.5 m above the ground respectively.

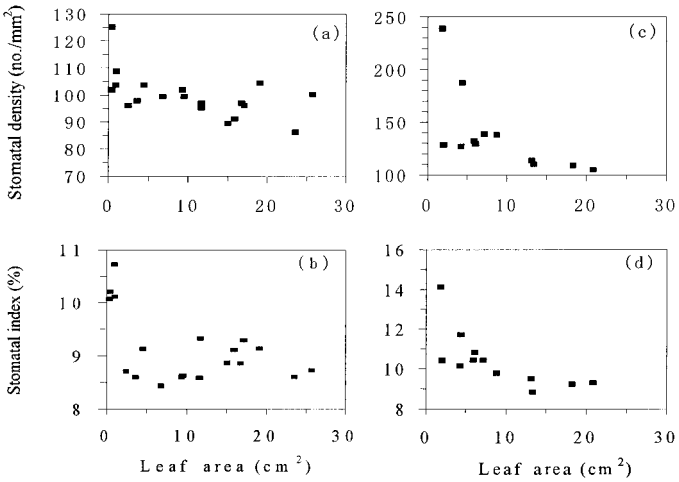


Fig. 12. The relationship between leaf area and stomatal density and index on short shoots (a and b) and on long shoots (c and d), respectively. Note the difference in *y*-axis scaling. Correlation details: (a) $r = -0.55$, $N = 19$, $P = 0.05$ (b) $r = -0.51$, $N = 19$, $P = 0.05$, (c) $r = -0.64$, $N = 12$, $P = 0.05$, (d) $r = -0.70$, $N = 12$, $P = 0.05$.

cant differences in stomatal index were detected for *S. herbacea* or *G. biloba*, which tends to confirm the view that stomatal index is more stable than stomatal density.

The response of stomatal density and index of G. biloba leaves to atmospheric CO_2 change—The mean stomatal density of the herbarium *G. biloba* leaves collected in 1924 was 134 stomata/mm² and compares with the current value of 97 stomata/mm² (Table 3). The values of stomatal density and index of modern *G. biloba* growing in the 1998 atmospheric CO_2 concentration in Table 3 were calculated excluding the small (<2 cm²) leaves considered in Fig. 12. Kanis and Karstens reported in 1963 that the stomatal density of *G. biloba* trees growing in the Netherlands ranged between 110 and 140 stomata/mm². Over this 74-yr period, ice core (Friedli et al., 1986) and instrumental (Keeling and Whorf, 1994) records indicate that the global atmospheric CO_2 concentration has increased by 55 μmol/mol. Collectively therefore these data indicate a response consistent with an historical CO_2 effect on stomatal development at this time, an effect that has also been observed in CO_2-enrichment experiments with *G. biloba* (Beerling, McElwain, and Osborne, 1998).

The stomatal density and index of three species from the Jurassic and one species from the Early Cretaceous were investigated to test for evidence of the stomatal CO_2 response in the plant fossil record (Table 3). We assume with the fossil material that from the very fact of their presence in the fossil record (i.e., as organs shed from the parent tree), coupled with the cuticle being robust enough for preservation, we were dealing with mature leaves. In all cases, the leaf stomatal density and index of these four species were significantly lower than the values for extant *G. biloba* ($P < 0.05$) (Figs. 5–9, Table 3), which suggests that there was a relatively high atmospheric CO_2 concentration in the Mesozoic.

TABLE 2. Mean stomatal density and index of leaves of female and male *Ginkgo biloba* trees (N = number of leaves, i.e., five leaves on each of five trees).

Tree type	Stomatal density			Stomatal index		
	Mean	SE	*N*	Mean	SE	*N*
Female tree	95.6	0.5	25	9.4	0.01	25
Male tree	84.5	0.4	25	9.4	0.02	25

From these data (Table 3) we next calculated the stomatal ratios of the fossils (defined as the ratio of the fossil plant stomatal index relative to that of modern *G. biloba*). These were then converted to RCO_2 values (defined as the ratio of atmospheric CO_2 in the past relative to the preindustrial value of 300 μmol/mol) following the standardization procedure of McElwain (1998) to allow comparison with the predictions of a long-term carbon cycle model (Berner, 1994, 1998).

Calculated in this way, the stomatal ratios of *G. coriacea* from Early Cretaceous and *G. yimaensis* from early Middle Jurassic agree closely with Berner's CO_2 curve, while the stomatal ratios of *G. obrutschewii* and *G. huttoni* from the Early and Middle Jurassic are somewhat lower (Fig. 13). Remarkably, the stomatal ratios of the last two species correspond to the troughs of atmospheric CO_2 concentration at those points on the time axis of Berner's curve (Fig. 13). Earlier work on plant fossils has indicated a fourfold increase in atmospheric carbon dioxide at the Triassic–Jurassic boundary (McElwain, Beerling, and Woodward, 1999), after which the atmospheric CO_2 falls again. The stomatal ratio of *Baiera spectabilis* from Jameson Land (depth 20 m equal to the Early Jurassic), East Greenland is only 1.69 (McElwain, Beerling, and Woodward, 1999), which is quite close to the stomatal ratio of *G. obrutschewii* (1.67) from the Early Jurassic.

General conclusions—Our measurement on herbarium and fossil materials, and the results of earlier CO_2 enrichment experiments, are all consistent with the evidence that leaves of plants of *Ginkgo* are sensitive to changes in the atmospheric CO_2 concentration (Table 3). The stomatal density and index

TABLE 3. Stomatal characters of *Ginkgo* leaves of different ages.

Species	Age	Stomatal density			Stomatal index			Stomatal ratio
		Mean	SE	*N*[a]	Mean	SE	*N*	
Ginkgo biloba	1998	97.7	0.1	(140)	9.3	0.01	(140)	
G. biloba	1924	134.0	3.7	35(7)	n/d[b]	n/d[b]	35(7)	
G. coriacea	Early Cretaceous	20.3	0.8	90	3.4	0.1	90	2.8
G. huttoni	Middle Jurassic	44.8	1.2	52	5.5	0.1	52	1.7
G. yimaensis	Early Middle Jurassic	10.1	0.4	23	2.6	0.1	7	3.6
G. obrutschewii	Early Jurassic	59.3	0.7	28(3)	6.7	0.04	28(3)	1.4

[a] N = The number of counts on fossil cuticles from bulk macerated *Ginkgo* leaves. The exact number of source leaves is uncertain except where noted by a number of leaves in parentheses.

[b] n/d = no data. The replicas of herbarium material did not reveal epidermal cell outlines with sufficient clarity to yield stomatal index values.

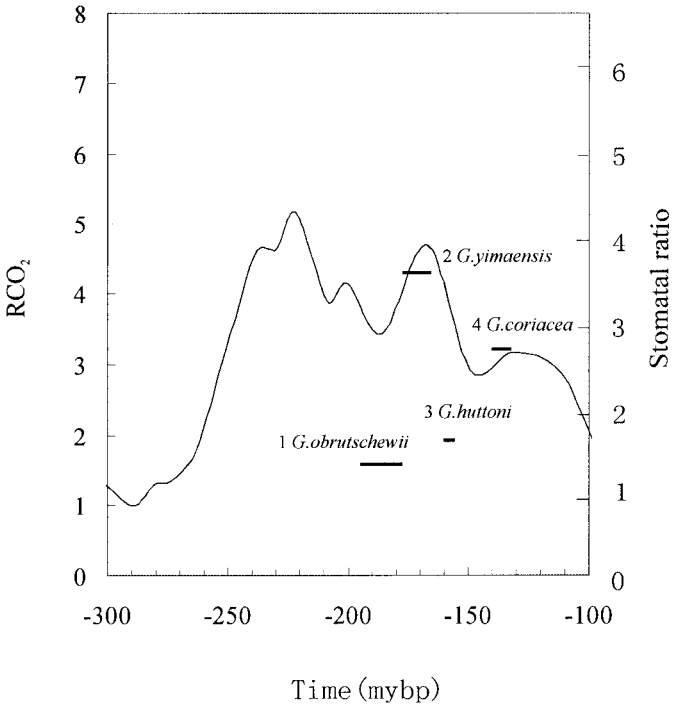


Fig. 13. The ''best guess'' CO_2 curve (solid line) predicted by a long-term global carbon cycle model (Berner, 1994, 1998), where RCO_2 is a ratio of CO_2 concentration in the past relative to the preindustrial value, and the stomatal ratio is calculated from the ratio of the stomatal index of the fossils leaves (Table 3) to the stomatal index of living Ginkgo (horizontal lines). The length of the horizontal lines denotes the age uncertainty associated with the fossil materials using the time scale of Harland et al. (1989). mybp = −300∼−100.

of mature modern *G. biloba* leaves show some variation with respect to date of sampling (Fig. 10), position in the canopy (Fig. 11), and shoot type (Table 1). However, critically, we found that while this variation was significant in some instances, the difference was considerably less than the observed difference in leaf stomatal density and index between modern and fossil samples. Moreover, the variation in RCO_2 through the past 200 million years reconstructed from fossil leaves is generally consistent with the predictions of a long-term carbon cycle model. Very young immature leaves often show both high stomatal density and stomatal index (Fig. 12). Hence, mature leaves are the only material that should be used for studies on the relationship between atmospheric CO_2 concentration and leaf stomatal characters.

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