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Fossil plant stomata indicate decreasing atmospheric CO_2 prior to the Eocene–Oligocene boundary

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Abstract

A unique stratigraphic sequence of fossil leaves of *Eotrigonobalanus furcinervis* (extinct trees of the beech family, Fagaceae) from central Germany has been used to derive an atmospheric pCO_2 record with multiple data points spanning the late middle to late Eocene, two sampling levels which may be earliest Oligocene, and two samples from later in the Oligocene. Using the inverse relationship between the density of stomata and pCO_2, we show that pCO_2 decreased continuously from the late middle to late Eocene, reaching a relatively stable low value before the end of the Eocene. Based on the subsequent records, pCO_2 in parts of the Oligocene was similar to latest Eocene values. These results show that a decrease in pCO_2 preceded the large shift in marine oxygen isotope records that characterizes the Eocene–Oliogocene transition. This may be related to the "hysteresis effect" previously proposed – where a certain threshold of pCO_2 change was crossed before the cumulative effects of this and other factors resulted in rapid temperature decline, ice build up on Antarctica and hence a change of climate mode.

1 Introduction

1.1 The role of pCO_2 in Cenozoic climate

The Cenozoic era is characterized by large climatic variations, including the fundamentally important transition from an ice-free "greenhouse" planet to the modern "icehouse" planet with polar glaciations. This climatic transition is generally thought to have been driven primarily by changes in pCO_2 and/or the thermal isolation of Antarctica by the opening of Southern Ocean gateways (DeConto and Pollard, 2003; Zachos et al., 2008; Hansen et al., 2013; Hren et al., 2013; Goldner et al., 2014; Inglis et al., 2015). However, the full extent of the role of pCO_2 in Cenozoic climate change remains unresolved. The most detailed Cenozoic temperature and pCO_2 records are derived from marine isotope proxies (e.g. Foster et al., 2012; Pagani et al., 2011; Pearson et al., 2009; Zachos et al., 2001, 2008). Isotope records, however, may be influenced by a variety of taphonomic and diagenetic biases (see Coxall and Pearson, 2007 for review; and Pagani et al., 2011), that can obscure the climatic signal, and thus need independent evaluation by separate proxy records (Berling and Royer, 2011).

Eocene temperatures were globally much higher than today, leading to a weakened equator-to-pole temperature gradient and muted seasonal cycle compared to today; the so-called "Eocene equable climate problem" (Sloan and Barron, 1992; Huber and Caballero, 2011). Climate modelling has been able to reconstruct this climatic pattern only with excessively high pCO_2 levels ($\sim$ 4500 ppm: Huber and Caballero, 2011), but such elevated pCO_2 atmospheres do not agree with most proxy records. It has therefore been speculated that Eocene climate sensitivity was elevated compared to today and/or that other forcing in addition to high pCO_2 was involved (Caballero and Huber, 2013; Hansen et al., 2013). In order to solve this enigma reliable multiple proxy records of pCO_2 are of paramount importance.

The fundamental climatic reorganization that occurred close to the Eocene–Oligocene boundary (33.8 Ma), often referred to as the Eocene–Oligocene transition (EOT, 34–33.5 Ma), had drastic consequences for biological systems. These included both terrestrial and marine faunal and floral extinctions accompanied by evolutionary turnover (Prothero, 1994; Coxall and Pearson, 2007; Sheldon et al., 2009: Kunzmann, 2012; Kvaček et al., 2014), although vegetation changes in the European terrestrial record appear to be less dramatic and more gradual (Kvaček et al., 2014; Kunzmann et al., 2015). General circulation models of Paleogene climate have shown that continuously declining pCO_2, amplified by Milankovitch forcing and ice-albedo feedbacks, could cause significant temperature reduction. This could result in a permanent continental Antarctic ice-sheet once a critical pCO_2 threshold, generally considered to be $<$ 700 ppm is crossed e.g. (DeConto and Pollard, 2003; Coxall et al., 2005; Pollard and DeConto, 2005; Zachos and Kump, 2005; Pagani et al., 2011; Hansen et al., 2013). Recently, based climate modelling, the timing of the Eocene–Oligocene cooling

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has been re-evaluated, with new results indicating that rather than a sudden cooling episode close to the Eocene–Oligocene boundary, pCO_2 decrease and subsequent substantial cooling took place gradually during the mid-late Eocene (Goldner et al., 2014; Inglis et al., 2015).

Four proxies have been identified as being particularly useful for Cenozoic pCO_2 reconstructions by the Intergovernmental Panel on Climate Change (initially reported in the 4th IPCC report, 2007), including the terrestrial proxy based on stomatal densities of fossil plants. Previous studies using the stomatal proxy method of pCO_2 reconstructions for the part of the Cenozoic relevant here were, however, mostly of low resolution and have been inconclusive. Some suggested that pCO_2 was essentially stable at between 300 and 450 parts per million by volume (ppm) during the Eocene, Oligocene and Miocene (Royer, 2001; Royer et al., 2001) and others suggesting distinct decrease in pCO_2 across the Eocene–Oligocene boundary (Retallack, 2001). More recent studies suggest higher and possibly rapidly decreasing pCO_2 (ranging ca. 1000–500 ppm) during the late middle Eocene (Doria et al., 2011; Grein et al., 2011). One study suggests that pCO_2 was significantly higher at the EOT than during the early Oligocene (Roth-Nebelsick et al., 2004) and others that early Oligocene to early Miocene pCO_2 was ca. 400 ppm throughout (Grein et al., 2013; Roth-Nebelsick et al., 2014).

Here we present a new stomatal proxy-based record with multiple data points spanning the late middle to late Eocene, two sampling levels that according to current available evidence are from the earliest Oligocene, and two samples from later in the Oligocene. The record is based on a large dataset of plant leaf stomatal densities of a single fossil species – considered ideal when employing this proxy method. The mechanisms behind and specific approaches involved in the stomatal proxy method are introduced in more detail below.

1.2 The stomatal proxy method of paleo-pCO_2 reconstruction

Stomata are pores on plant leaf surfaces through which gas exchange takes place; i.e. carbon is obtained from CO_2 and at the same time water vapour and oxygen are

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lost by diffusion. An inverse relationship exists between the frequency of stomata and pCO_2, due to optimization of gas exchange by plants, which results in plants preserving water by reducing the number of stomata when CO_2 is readily available. Woodward (1987) established this from observations of herbarium material, showing that modern tree species have responded to the anthropogenic rise in pCO_2 by reducing their stomatal frequency significantly. The inverse relationship between stomatal frequency, recorded as "stomatal density" (SD = the number of stomata per mm^2) or "stomatal index" (SI = the percentage of stomata relative to epidermal cells), and pCO_2 has been repeatedly demonstrated for a wide variety of plant taxa from disparate geological and ecological settings from the Palaeozoic until today and is thus established as a strong proxy for paleo-pCO_2 (e.g. Beerling et al., 1998; McElwain, 1998; Retallack, 2001; Royer et al., 2001; Kürschner et al., 2008; Steinthorsdottir et al., 2011b, 2013; Steinthorsdottir and Vajda, 2015). The increasingly close match between stomatal proxy pCO_2 results and independent proxy records, actual pCO_2 measurements and in some cases climate modelling (e.g. Finsinger and Wagner-Cremer, 2009; Foster et al., 2012; Kürschner et al., 2008; Retallack, 2001; Rundgren and Björck, 2003; Steinthorsdottir and Vajda, 2015) instils growing confidence in stomatal frequency for recording past pCO_2. Strongly supporting the validity of the stomatal proxy is also the identification of the mechanism by which plants control their stomatal densities based on atmospheric pCO_2. All plants use the enzyme carbonic anhydrase to detect pCO_2 around their leaves (Frommer, 2010; Hu et al., 2010); mature leaves (early shoots) then control stomatal development of younger leaves through long-distance signalling (Lake et al., 2002), involving the HIC gene signalling pathway (Brownlee, 2001; Gray et al., 2000).

In order to transform stomatal frequency data derived from fossil plants into paleo-pCO_2 estimates it is usually necessary to compare stomatal data from present day plants that are either phylogenetically related or in other ways equivalent to the fossil plants. Nearest living relatives (NLR) should be used when possible, but when these cannot be identified for the fossil plants, nearest living equivalents (NLE = present day

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species that are of comparable ecological setting and/or structural similarity to their fossil counterpart) may be used instead (McElwain and Chaloner, 1995; Barclay et al., 2010; Steinthorsdottir et al., 2011a, b).

There are three stomatal paleo-pCO_2 calibration methods in use. These are (i) the "stomatal ratio method" (McElwain and Chaloner, 1995; McElwain, 1998), which relies on a ratio between stomatal frequencies of fossil plants and their NLE to semi-quantify pCO_2; (ii) the "transfer function method", which relies on herbarium material and/or experimental datasets for NLR/NLE responses to calculate pCO_2 curves (e.g. Beerling and Royer, 2002); and (iii) the more recently developed taxon-independent "mechanistic gas exchange modelling" approach (e.g. Franks et al., 2014; Grein et al., 2013; Roth-Nebelsick et al., 2014) which, at its core, uses measurements of stomatal density and pore size to estimate maximum theoretical gas exchange rates, from which paleo-assimilation rate and pCO_2 are simultaneously estimated by iteration. The stomatal ratio method, which is used here, calibrates paleo-pCO_2 based on two so-called standardizations. The first is the "modern" standardization that assumes that the ratio between past and modern pCO_2 is 1 ($RCO_2 = 1$) and is applied to young material, typically from the Quaternary. The second is the "Carboniferous" standardization that sets the ratio between past and modern pCO_2 at two times preindustrial levels of 300 ppm ($RCO_2 = 2 = 600$) (McElwain and Chaloner, 1995). Both standardizations are usually applied to fossil leaf material of Cenozoic age and older to yield minimum and maximum pCO_2 estimates and both standardizations will be used in this paper.

2 Material and methods

2.1 Fossil leaf database

Eotrigonobalanus furcinervis (Rossm. 1840) Walther et Kvaček in Kvaček and Walther (1989), an extinct evergreen Fagaceae, (Fig. 1) existed from the middle Eocene to the Oligocene–Miocene boundary and was geographically widely distributed, i.e. from central Europe to Russia, as well as to the Mediterranean area (Mai and Walther, 2000; Velitzelos et al., 1999). It is considered as a thermophilous species that grew in evergreen broadleaved forests as well as in mixed mesophytic forests adapted to humid and warm-temperate to subtropical climate (Mai and Walther, 2000). *E. furcinervis* was present in megafossil assemblages or "taphocoenoses" derived from riparian forests, back swamps, peat bogs and zonal vegetation and therefore the parent plant tolerated a wide range of water table conditions and soil characteristics. Whereas in the Eocene it often predominated in zonal Fagaceae–Lauraceae forests (Mai and Walther, 2000), in the Oligocene mixed mesophytic forest it was ecologically sub-dominant. Based on the combined fossil record of cupules, seeds and leaves, including cuticles, it is commonly accepted that the fossils represent a single long-lived but rather variable fossil species, although minor changes in leaf anatomy have led to the distinction of two subspecies, ssp. *furcinervis* (mainly Eocene, rare in Oligocene) and ssp. *haselbachenses* (only Oligocene; Kvaček and Walther, 1989). The latter is distinguished by the absence of pubescence (trichome clusters) on the abaxial leaf epidermis. Furthermore, a variety of leaf morphotypes can be distinguished that have been interpreted as ecological variants (ecotypes, see Kriegel, 2001).

Except for the material from the Kleinsaubernitz site (Fig. 2), the leaf specimens used here originate from the central German Weißelster Basin (Fig. 2), a coastal alluvial plain at the southern margin of the North German–Polish "Tertiary" Basin (Standke, 2008). This basin is well-known for its extensive record of middle Eocene to early Miocene plant assemblages that are mainly derived from azonal vegetation, i.e. riparian and swamp forests (e.g. Mai and Walther, 2000; Kunzmann, 2012). The Knau assemblage represents the fluvial hinterland of the Weißelster lignite swamps (Mai and Walther, 2000).

The leaves used here are derived from a succession of cuticle rich taphocoenoses that contain *E. furcinervis* ranging in age from the late middle Eocene to the end of the Oligocene (Table 1, Fig. 3). The database analysed here consists of 233 *E. furcinervis* leaf cuticle fragments on as many slides, representing 151 separate individual leaf specimens (Supplement and Table 2). All specimens represent material used in previous taxonomic-systematic studies, they are housed in the Senckenberg Natural History Collections Dresden, Germany. The plant fossil assemblages have been positioned on the most recent lithostratigraphy for central and East Germany (Standke, 2008; Standke et al., 2010; Fig. 3, Table 2) using published information on the fossil sites (Mai and Walther, 1991, 2000; Kunzmann and Walther, 2002; Hennig and Kunzmann, 2013; Ferdani, 2014) and personal observations (LK). Information on dating is provided in Sect. 2.2 below.

One late Oligocene locality, Kleinsaubernitz (Figs. 2 and 3a), lies within the Lausitz basin, at its southern margin or even in the hinterland (Standke, 2008). Leaf specimens derive from a sediment-filled maar, volcanic in origin, preserving a parauthochtonous assemblage mainly representing zonal vegetation (Walther, 1999) in contrast to the mainly azonal vegetation from the coastal plains of the Weißelster Basin.

2.2 Stratigraphy and dating

The relative stratigraphic positions for the samples from the Weißelster Basin (Figs. 2 and 3a) are based on accumulating knowledge from more than 150 years of geological-paleontological investigations of the respective units (see Walther and Kunzmann, 2008 for summary). The samples are derived from a superposed sequence of four lignite seams and their associated strata (Table 1, Fig. 3a) the subdivisions of which can be readily recognized across different opencast mines.

It is not possible to directly correlate the plant fossil bearing horizons in the Weißelster Basin to the global marine stratigraphy. Although there are a number of brackish-marine intercalations (Standke et al., 2010) most of these strata lack fossils suitable for biostratigraphy. As is typical for lignite-bearing non-consolidated sedimentary successions (i.e. gravel, sands, silts, clays) hard parts of mineralised organisms that might be used for biostratigraphy in continental sequences (such as mammals and charophytes) are lacking due to dissolution by humic acids originating from organic material. Non-consolidated sediments do not reveal any casts or molds of these former fossils.

This is also the case for any intercalation of brackish-marine sediments in the Weißelster Basin profile. The lack of common index fossils prevents accurate stratigraphic chronology in the basin and reduces the level of stratigraphic resolution compared with that typically attainable for marine deposits (e.g. Roth-Nebelsick et al., 2014). Furthermore, heterogeneity in facies types (channel, floodplain, tidal deposits, swamps) and in grain sizes of the sediments precludes the use of magnetostratigraphic methods which need longer sequences of fine-grained sediments without facies shifts (e.g. lake sediments) to produce reliable data.

Based on a series of consecutive pollen assemblages in the Weißelster Basin strata a regional phytostratigraphic concept was developed (Krutzsch, 1967) that can be applied to all Formations, Members and submembers, and also to all lignite seams and even individual seam measures (Krutzsch, 2011). All of our investigated material is unambiguously assigned to a certain unit of the regional lithostratigraphic scheme (Fig. 3a) and thus connected to a respective pollen zone or subzone (Fig. 3a, Table 1). However, the pollen zonation yields only a relative age for a given horizon within the regional palynostratigraphic framework and does not enable correlation to global stratigraphy or to the global time scale. The attempt by Krutzsch (2011) to correlate the Eocene spore-pollen zones with the global timescale is used herein (Fig. 3a) as it is the only available information to interpret our assemblages. A "late" Eocene age (i.e. late Bartonian + Priabonian, Krutzsch, 2011) for our respective assemblages has been previously inferred based on floristic comparison to assemblages from the nearby Bohemian basins (Czech Republic) some of which have absolute dates from volcanic rocks (i.e. Kučlin, Staré Sedlo, Roudníky; Kvaček et al., 2014).

In the younger part of the succession, marine deposits have yielded index fossils suitable for biostratigraphy. Marine strata above the Gröbers Member of the Böhlen Formation are placed into regional dinoflagellate zones D13 and D14 (Köthe, 2005; Standke et al., 2010) which are Rupelian in age. The Haselbach horizon of the Gröbers Member, including our assemblage sites Schleenhain 4 and Haselbach 2 (Figs. 2 and 3a), was therefore interpreted to be basalmost Oligocene (Standke et al., 2010; Krutzsch,

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2011), however, the only definitive information from the dinoflagellate data is that the samples must be older than mid Rupelian. Lithofacies changes in the centre of the Weißelster Basin, i.e. the profile in the Schleenhain mine (Kunzmann and Walther, 2002) that indicate major sea level changes below the sample horizon of sites Schleenhain 4 and Haselbach 2 are consistent with those that occur around the Eocene – Oligocene boundary and are documented in other European successions (e.g. Hooker et al., 2009). A basalmost Oligocene age for the Schleenhain 4 and Haselbach 2 sites is also indicated by the first occurrence of *Boehlensipollis hohlii* in the sampled horizon which places the sample in spore-pollen zone 20 A/B sensu Krutzsch (2011). *Boehlensipollis hohlii* is regarded as a key element for the Oligocene in central and East Germany (Krutzsch, 2011) and had also been treated as such in the International Geological Correlation Programme (Vinken, 1988). However, it should be mentioned that Collinson (1992) reported several occurrences of the species in the late Eocene of the UK and Frederiksen (1980) reported the species ranging from late middle Eocene to Oligocene in the USA. Possibly the species arose in the USA and spread later via the UK into central Europe but further work is needed to securely link the occurrences of *Boehlensipollis hohlii* with the marine biostratigraphy and the global time scale. In short, there are two independent pieces of evidence (lithofacies, first appearance of *Boehlensipollis hohlii*) that clearly suggest an early Oligocene age for the Schleenhain 4 and Haselbach 2 samples. However, this is not conclusive evidence and direct linkage to the global marine scale is currently not available. The site at Kleinsaubernitz has been located on Fig. 3 based on its pollen assemblage which is zone 20 G (Goth et al., 2003).

In summary, the material from the Weißelster Basin comes from a superposed sequence where relative stratigraphic position is securely known (Table 1). Relative changes of SD (and thus pCO_2) through the succession can be placed in context of the spore-pollen zonation. However, the positions of the Eocene–Oligocene boundary and the Oligocene–Miocene boundary cannot be located with certainty in the Weißelster profiles. All age estimates in Figs. 3 and 4 are based on Krutzsch's (2011) proposed

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correlation of the regional spore-pollen zones to global sea level changes. Independent support is needed for these proposals so they should be regarded as preliminary age information.

2.3 Stomatal density quantification

Cuticles were prepared at the Senckenberg Natural History Collections Dresden as a part of an existing collection. Cuticle slides were prepared using standard methods for Paleogene material. Fragments removed from leaf specimens with preparation needles were macerated for 1–4 min in Schulze's solution. Cuticles were then neutralized with NH_4OH, washed with distilled water, and upper and lower cuticles were separated using preparation needles. Finally, the cuticles were stained with Safranin and affixed to slides by glycerol jelly. For this study, the slides were examined microscopically by an adaptation of the methodology set out by Poole and Kürschner (1999) in order to determine SD. According to this protocol, counts from mid lamina are preferable in establishing SD, but the fragmented nature of a proportion of the fossil material did not allow establishing where individual cuticle samples were located on the original leaf surface (see Fig. 1b). Individual epidermal cells were not easily discernible in the majority of the *E. furcinervis* material, making SI determination impossible. SD was obtained using a Nikon SK Light Microscope at ×200 magnification with a graticule providing a counting field of 0.042 mm^2. The graticule was centred over areas where stomata occurred in greatest numbers (away from veins and margins where those were known, *sensu* Poole and Kürschner, 1999) and up to five individual counts were recorded for each slide, resulting in 659 SD counts for the database of 151 leaf specimens (Table 1 and Supplement). Data was stored in Microsoft Excel 2010 before being statistically manipulated using MINITAB (version 16.1.1 for Windows).

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2.4 Choice of Nearest Living Equivalent and Paleo-pCO_2 calibration

Eotrigonobalanus furcinervis belongs to the Fagaceae, but its phylogenetic position is not well defined. Based on cupule morphology, *Eotrigonobalanus* belongs to a basal clade of the family, exhibiting intermediate characters between modern *Trigonobalanus* and *Castanopsis* (Mai, 1995). However, leaf venation and leaf cuticle micromorphology place *Eotrigonobalanus* with *Trigonobalanus* and *Lithocarpus,* away from *Castanopsis* (Kvaček and Walther, 1989), an affiliation recently confirmed by Denk et al. (2012). Since the phylogeny of Fagaceae has changed considerably (Manos et al., 2001, 2008), an improved systematic framework is still required to confirm the phylogenetic position of *Eotrigonobalanus*. Because the exact relationship of *Eotrigonobalanus* to crown group Fagaceae is unknown, a single nearest living relative (NLR) could not be obtained, hence the nearest living equivalent (NLE) approach has been used for the stomatal proxy-based pCO_2 reconstruction.

In this study, *Trigonobalanus doichangensis* was chosen as NLE, due to it being a basal species within the Fagaceae family and having leaf macro-morphological and leaf cuticle micro-morphological similarities with *E. furcinervis*, including cyclocytic stomata and similarly structured trichomes (Kvaček and Walther, 1989; see also Denk et al., 2012). Two herbarium specimens of *T. diochangensis,* formerly collected in 1988, were sampled at the Kew Herbarium (Royal Botanical Gardens, Kew, Richmond, Surrey, UK). Approximately 1 cm^2 was cut from mid-lamina of each leaf specimen and dry mounted onto a slide. Five cuticle images from each slide were taken at 200× magnification using a Leica DM2500 epifluorescent microscope with Leica DFC300FX camera (Leica® 312 Microsystems, Wetzlar, Germany) and Syncroscopy Automontage (Syncroscopy Ltd, Cambridge, UK) digital imaging software. A 0.09 mm^2 square was superimposed on each image and stomatal density was determined within this square following the protocol of Poole and Kürschner (1999). SD was determined to be 546.11 mm^{-2} at pCO_2 of 351 ppm (collection year levels according to NOAA ESRL data, available at www.esrl.noaa.gov).

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Using the stomatal ratio method with *T. doichangensis* NLE for *E. furcinervis*, we calibrated paleo-pCO_2 using the equations below to derive minimum and maximum paleo-pCO_2 ("Modern" and "Carboniferous" Standardization of McElwain and Chaloner, 1995), respectively:

$$\text{Paleo-}pCO_{2\,\text{min}}\ (\text{ppm}) = ((SD_{NLE} = 546.11)/SD_{fossil}) \times 351\,\text{ppm}$$

$$\text{Paleo-}pCO_{2\,\text{max}}\ (\text{ppm}) = ((SD_{NLE} = 546.11)/SD_{fossil}) \times 600\,\text{ppm}$$

3 Results

3.1 Stomatal density and paleo-pCO_2 estimates

SD of *E. furcinervis* range between ca. 425 and 740 stomata mm^{-2}. The lowest SD values (signifying highest pCO_2) are found in the oldest deposits, late middle to earliest late Eocene (spore-pollen zone 17), and the highest values (signifying lowest pCO_2) are found in the later late Eocene (spore-pollen zone 18o), representing the most pronounced SD change during the time period covered by the dataset (Table 2, Fig. 3b), with three intermediate samples showing intermediate values (spore-pollen zones 17/18, 18 u, 18 uo). During this interval SD increases by > 300 stomata mm^{-2} or by ca. 75 %, a very significant change indicating a sizeable decrease in pCO_2 in perhaps ca. 3.5 million years. Stomatal densities then decrease slightly again and remain around 600–650 mm^{-2} in the latest Eocene and in samples that may be earliest Oligocene as well as in the late Oligocene (spore-pollen zones 19, 20 A/B, 20 G, II). At the end of the Oligocene, SD decreases again to ca. 570 mm^{-2}.

Paleo-pCO_2 calibrated using the stomatal densities of *E. furcinervis* will be discussed as average values and evaluated in terms of relative change, as introduced above. The largest change in paleo-pCO_2 is the decrease from the late middle to earliest late Eocene of > 250 ppm, from ca. 630 ppm to ca. 365 ppm – a decrease in pCO_2 of ca.

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40 % (Fig. 3b; Table 2). Concentrations of CO_2 then increase again by ca. 45 ppm to ca. 410 ppm in the latest Eocene and possibly earliest Oligocene, and further to between ca. 430–475 ppm in the late and latest Oligocene (Fig. 3b; Table 2).

4 Discussion

4.1 Fidelity of the Saxony stomatal pCO_2 record

The Saxony fossil leaf database is unique in that this relatively large database derives from a well-constrained stratigraphic succession and consists of a single species throughout – *E. furcinervis* – which is the most ideal situation when using fossil leaf material to reconstruct paleo-pCO_2, since inter-species variability is eliminated and stomatal responses to pCO_2 are likely to be consistent through time. The procurement of a single-species dataset from multiple stratigraphic levels across several million years is not common, in particular when the stratigraphy represents time intervals of significant climate and/or environmental change, as is the case here. The principal challenge concerning the Saxony stomatal density record was translating the stomatal signal into reliable levels of pCO_2. One of the main limitations associated with the use of paleo-proxies is the preservational state of fossil material and in this case the preservation of fossil leaves did not allow paleo-pCO_2 reconstruction using gas exchange models for independent comparison of the results using the stomatal ratio method because stomatal pore length could not be measured in all samples with confidence. Additionally, there is a lack of available transfer functions for potential NLEs of *E. furcinervis*, so it was not possible either to obtain independent pCO_2 reconstructions using the transfer function method. The stomatal ratio method has however been shown to closely match results produced with transfer function methods (Beerling and Royer, 2002; Barclay et al., 2010; Steinthorsdottir et al., 2011b) and is seen as a good alternative where detailed estimates of other photosynthetic parameters, which are required to initialize mechanistic models, are not readily available (McElwain, unpubl.).

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The absence of an obvious NLE for *E. furcinervis* – an extinct species of uncertain phylogenetic affinity – further introduces potential errors in pCO_2 calibration. Although we consider *T. doichangensis* the best available NLE, there is no guarantee that its stomatal density and degree of response to pCO_2 closely mirrors that of its distant fossil relative. The pCO_2 levels calibrated here appear somewhat low compared to most previously published pCO_2 datasets, although broadly comparable to stomatal pCO_2 records (Fig. 4a). When testing three additional potentially suitable NLE species for reconstructing pCO_2 using the Saxony database; *Trigonobalanus verticillata, Castanopsis cuspidata* and *Lithocarpus henryi,* the resulting paleo-pCO_2 values were extremely low – considerably lower than when using the chosen NLE *T. doichangensis* – in many cases being lower than minimum pCO_2 levels required to maintain sufficient plant growth and reproduction (i.e. below the ecological compensation point). This indicates that, for some reason, the stomatal proxy-derived pCO_2 estimates presented here are likely artificially low and should probably all be adjusted uniformly upwards until this calibration problem is solved. This problem is by no means limited to this study, as will be discussed below.

4.2 Comparison with vegetation and proxy continental climate records

Paleoclimate reconstructions based on Central European megafloras reveal a sharp decline in continental cold month mean temperature (Mosbrugger et al., 2005) and mean annual temperature (Moraweck et al., 2015; Kvaček et al., 2014) in the late Eocene (Fig. 4b) which is consistent with the timing of the pCO_2 decline that we report here (Figs. 4a and b), but less so with global sea surface temperature trends as recorded by marine oxygen-isotopes (Fig. 4c). Although the marine isotope curve also shows a gradual decrease in the late Eocene, the most pronounced and abrupt change in temperatures coincides with the Eocene–Oliogocene boundary (Fig. 4c), suggesting that pCO_2 drawdown took place gradually before global temperatures reacted suddenly. Furthermore, paleo-vegetation analysis of the Weißelster and North Bohemian basins reveals that gradual restructuring of dominantly evergreen forests by immigra-

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tion of deciduous species such as *Platanus neptuni, Trigonobalanopsis rhamnoides* and *Taxodium dubium* (Kunzmann et al., 2015) took place in the late Bartonian to early Priabonian interval around ca. 38 Ma (Kvaček, 2010; Teodoridis and Kvaček, 2015). The temporal coincidence of pCO_2 decline and major vegetation transition – from angiosperm-dominated notophyllous evergreen forests to mixed mesophytic forests – suggests a potential causal role of pCO_2 decline in the changing ecological composition of forests. It may have been in part triggered by differential responses of evergreen and deciduous taxa to declining pCO_2 (Fig. 4a and b), explaining the lag between "temperatures" indicated by terrestrial vegetation and sea surface temperatures recorded by marine oxygen-isotopes (Fig. 4c). The functional trait of deciduousness is an adaptation to episodic cooling (Zanne et al., 2014). However, it has also been demonstrated experimentally (McElwain et al., 2015) and on theoretical grounds (Niinemets et al., 2011) that taxa with low leaf mass per area or LMA (i.e. those that are deciduous or herbaceous) and high stomatal conductance have faster photosynthetic rates than evergreens at lower atmospheric pCO_2. In contrast, evergreens have higher responsiveness in terms of photosynthetic rates at elevated pCO_2 (Niinemets et al., 2011). A transition from elevated to lower CO_2 atmospheres would therefore favour the eco-physiology of deciduous or low LMA taxa over evergreen high LMA species. Further experimental investigation is now required to tease apart the relative importance of "CO_2 starvation" and increased temperature seasonality on the late Bartonian to early Priabonian vegetation transition.

4.3 Comparison with other pCO_2 records

Previously published stomatal proxy-based pCO_2 records from the part of the Cenozoic relevant to this paper do not always agree, but instead report highly elevated (McElwain, 1998; Doria et al., 2011; Grein et al., 2011; Smith et al., 2010), intermediate (Retallack, 2009) or similar to modern (Royer et al., 2001) pCO_2 for the Eocene. Similarly high variability in estimated pCO_2 levels exists for the Oligocene as well as the Miocene (Grein et al., 2013; Kürschner et al., 2008; Roth-Nebelsick et al., 2014; Royer

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et al., 2001). In a series of papers studying stomatal parameters of plants from the late Eocene to early Miocene of Saxony, some including analysis of *E. furcinervis*, an overall trend emerged of higher pCO_2 in the late Eocene, lower pCO_2 in the early Oligocene and intermediate, stable levels of pCO_2, showing no statistically significant variation, in the late Oligocene, at the Oligocene–Miocene boundary and in the early Miocene (Grein et al., 2011, 2013; Roth-Nebelsick et al., 2004, 2014). These trends are independently supported by flora-based climate proxies and partially reflect the global marine temperature data, in particular the cooling at the Eocene–Oligocene transition (Roth-Nebelsick et al., 2004, 2012; Zachos et al., 2001, 2008). When pCO_2 levels are quantified based on stomatal parameters in a mechanistic model (Konrad et al., 2008), concentrations ranging from 400–700 ppm, but most consistently around 400 ppm, are found throughout the studied intervals, notably despite changes in stomatal parameters (Grein et al., 2013; Roth-Nebelsick et al., 2014).

Using a rigorous generalized statistical framework, Beerling et al. (2009) revised previously published pCO_2 estimates based on *Ginkgo* and *Metasequoia* from the early Eocene and middle Miocene upwards by 150–250 ppm. Based on this revision, average stomatal proxy-based pCO_2 is 450–700 ppm in the Paleogene and 500–600 ppm in the Neogene (Beerling et al., 2009). Interestingly, the younger set of pCO_2 estimates was fully compatible to marine proxy data and modelling results (e.g. Pagani et al., 2005; Hansen et al., 2008), whereas the older set of estimates seemed to underestimate pCO_2 compared to the other approaches, even after the upwards revision of stomatal pCO_2 values (see Fig. 4 in Beerling et al., 2009). The seemingly more pronounced underestimation for pCO_2 values from Paleogene material is also found in the present study, where late middle to latest Eocene and possible earliest Oligocene samples yield pCO_2 values at the very low end, or lower than, previously published stomatal estimates. By contrast, values from the end Oligocene and early Miocene are in broad agreement with previous estimates (see Fig. 4a). However, Kürschner et al. (2008) indicated that an upwards correction of 150–200 ppm – a so-called “correction factor” –

was necessary also when reconstructing Miocene paleo-pCO_2 with two species from the Lauraceae family.

Pearson et al. (2009) reconstructed pCO_2 for the late Eocene to early Oligocene using the planktonic foraminifera boron isotope pH proxy and found that the main reduction in pCO_2 took place before the main phase of EOT ice growth (ca. 33.6 Ma: DeConto et al., 2008), followed by a sharp recovery to pre-transition levels and then a more gradual decline. Their results thus support the central role of declining pCO_2 in Antarctic ice sheet initiation and development and agree broadly with carbon cycle modelling (e.g. Merico et al., 2008). The quantitative estimates of pCO_2 varied greatly however, according to which d^{11}B value was used to derive pH, with geochemical models of the boron cycle suggesting a range of 37–39 ‰ for sea water (sw) d^{11}B during this time (Simon et al., 2006). The range of pCO_2 values spanned from ca. 2000–1500 ppm at the upper end and ca. 620–450 ppm at the lower end (Pearson et al., 2009). Recently published alkenone-based pCO_2 records found significantly declining pCO_2 before, as well as during, the Antarctic glaciation (EOT and earliest Oligocene), supporting the pCO_2 pattern of Pearson et al. (2009) and the role of pCO_2 as the primary forcing agent of Antarctic glaciation, consistent with model derived thresholds (Pagani et al., 2011; Zhang et al., 2013). The alkenone-derived dataset values are overall higher than those derived by stomatal densities, with late Eocene values of ca. 1000 ppm, minimum value of ca. 670 at 33.57 Ma and then gradual decline to ca. 350 ppm at the Oligocene–Miocene boundary.

In general therefore, Cenozoic stomatal proxy-based pCO_2 values, reconstructed using the available methods, tend to report consistently lower pCO_2 values than alkenone- or boron-based proxies as well as those from mass balance modelling. Nonetheless, it is noteworthy that most existing stomatal proxy-based pCO_2 records report a similar range of low pCO_2 values for this time interval and an internally consistent pattern is emerging for the Cenozoic (see Fig. 4a). The issue of stomatal proxy-based pCO_2 reconstructions almost exclusively showing considerably lower pCO_2 than other pCO_2 proxies is as yet unresolved. An important advance was made when it was

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demonstrated that Cenozoic pCO_2 estimates based on stomata should be adjusted upwards by 150–250 ppm to closely match the estimates based on separate (marine) pCO_2 proxies (Kürschner et al., 2008; Beerling et al., 2009). However, the fact remains that the now numerous Cenozoic pCO_2 records based on stomatal parameters from a range of woody plant species all indicate considerably lower pCO_2. Stomatal proxy-based pCO_2 records that are independently calibrated using different species/genera and families usually agree with one another and show Eocene–Miocene pCO_2 in the range of 300–800 ppm (Fig. 4a). Although this discrepancy between proxies needs to be better understood before significant reevaluation of the role of pCO_2 in Cenozoic climate change is warranted, it should not be a priori rejected that collectively stomatal proxy records may accurately indicate lower pCO_2 levels during the Cenozoic than previously assumed.

5 Conclusions

The new terrestrial stomatal proxy-based pCO_2 record presented here, derived from fossil leaves of *Eotrigonobalanus furcinervis* (extinct Fagaceae, beech tree family) from Saxony, Germany, spans the late middle Eocene to latest Eocene, with two sampling levels which are probably from earliest Oligocene, and two samples from later in the Oligocene. The record indicates that pCO_2 decreased continuously and gradually by ca. 40 % during the late Eocene, from ca. 630 ppm in the late middle Eocene to ca. 365 ppm in the late Eocene and ca. 410 ppm near the Eocene–Oligocene boundary. Late and latest Oligocene pCO_2 was slightly higher at around 430–475 ppm. The substantial late Eocene decrease in pCO_2 reported here is consistent with terrestrial records of vegetation change and reconstructions of coldest month mean temperatures. Although the pCO_2 values reported here may be artificially low, due to factors inherent to stomatal proxy-based calibration, they nonetheless broadly agree with the pCO_2 range of previously published Eocene–Miocene stomatal proxy records, indicating that Cenozoic pCO_2 may have been considerably lower than previously thought based on marine proxies. The Saxony record further strongly indicates that decrease in pCO_2 took place before the recorded decrease in global sea surface temperatures. These results lend support to the theory that pCO_2 drawdown, rather than continental reorganization, was the main forcer of the Eocene–Oligocene climate change, when a "tipping point" was reached in the latest Eocene, triggering the plunge of the Earth System into icehouse conditions, supporting the hysteresis hypothesis for this important climate change event.

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Table 1. Lithostratigraphic and phytostratigraphic positions of the *Eotrigonobalanus furcinervis*-containing fossil taphocoenoses in the Weißelster Basin (central Germany); lithostratigraphy after Standke et al., 2010, spore-pollen zonation after Krutzsch (2011).

Assemblage/site	Reference for fossil flora	Formation	Member	Horizon	Epoch	Spore-pollen zone
Witznitz	Mai and Walther (1991)	Cottbus	Thierbach	Witznitz	end of Oligocene	II
Espenhain-Störmthal	Mai and Walther (1991)	Cottbus	Thierbach	Witznitz	end of Oligocene	II
Haselbach 2	Mai and Walther (1978)	Böhlen	Gröbers	Haselbach	earliest Oligocene?	20 A/B
Schleenhain 4	Kunzmann and Walther (2012)	Böhlen	Gröbers	Haselbach	earliest Oligocene?	20 A/B
Schleenhain 3	Kunzmann and Walther (2002)	Borna	Domsen	overlying bed of lignite seam 23 o	latest Eocene	19 (?)
Schleenhain 2	Ferdani (2014); Mai and Walther (2000)	Borna	Bruckdorf	underlying bed of lignite seam 23 o and leaf measure in lignite seam 23 o	late Eocene	18o
Haselbach 1	Mai and Walther (2000)	Borna	Bruckdorf	intercalated bed between lignite seam 23 u and 23 o	late Eocene	18 uo
Schleenhain 1	Hennig and Kunzmann (2013)	Borna	Bruckdorf	overlying bed of lignite seam 23 u	late Eocene	18 u
Knau	Mai and Walther (2000)	Borna	uncertain	fluvial deposit	late Eocene	17/18
Profen-Süd	Fischer in Mai and Walther (2000)	Profen	Wallendorf	underlying bed of lignite seam 1	late middle Eocene	17

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Table 2. The Saxony *Eotrigonobalanus furcinervis* database, including spore-pollen zones (Krutzsch, 2011) and epoch inferred from them, stomatal density counts and pCO_2 calibration results.

Sites	Epoch	Spore-pollen zone	SD (stomata mm^{-2})	pCO_2 Min	pCO_2 Max	pCO_2 Average	No. of leaves
Witznitz, Espenhain-Störmthal	latest Oligocene	II	569.02 ± 108.40	351.6 ± 79.12	600.02 ± 135.03	**475.81** ± 107.08	45
Kleinsaubernitz	late Oligocene	20 G	623.29 ± 97.82	316.8 ± 58.41	540.71 ± 99.7	**428.76** ± 79.05	25
Schleenhain 4, Haselbach 2	earliest Oligocene (?)	20 A/B	657.13 ± 118.98	302.5 ± 59.31	516.29 ± 101.23	**409.40** ± 80.27	21
Schleenhain 3	latest Eocene	19	642.88 ± 84.05	303.1 ± 35.54	517.24 ± 60.66	**410.17** ± 48.10	11
Schleenhain 2	late Eocene	18 o	740.65 ± 148.90	269.56 ± 53.01	460.05 ± 90.48	**364.80** ± 71.74	39
Haselbach 1	late Eocene	18 uo	505.88± 47.06	373.50± 35.99	637.45± 61.43	505.48 ± 48.72	2
Schleenhain 1	late Eocene	18 u	661.18 ± 90.93	296.15 ± 44.65	505.43 ± 76.206	**400.79** ± 60.429	4
Knau	late Eocene	17/18	495.50 ± 77.80	397.33 ± 68.7	678.12 ± 117.25	**537.73** ± 92.98	4
Profen-Süd	late middle Eocene	17	426.14 ± 83.56	467.87 ± 101.78	798.51 ± 173.71	**633.19** ± 137.74	1
							Total:151

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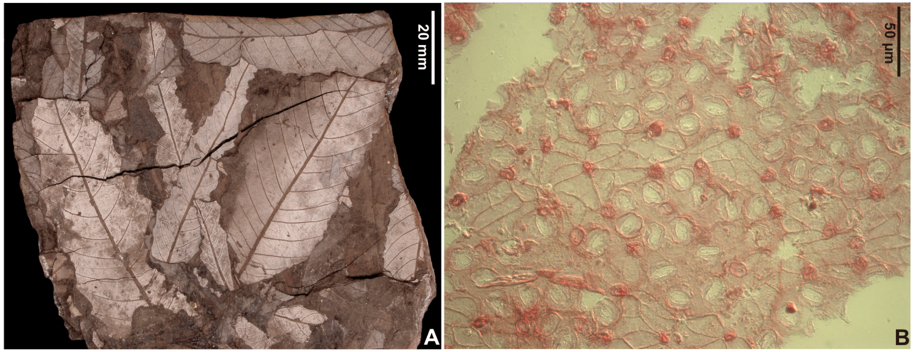

Figure 1. *Eotrigonobalanus furcinervis* (Rossm. 1840) Walther et Kvaček in Kvaček and Walther (1989), **(a)** mass occurrence of leaves in lignite, Schleenhain opencast mine, Saxony, Germany, site Schleenhain 2, Borna Formation, Bruckdorf Member, late Eocene (Priabonian), SPP zone 18o, MMG PB SchleOE 535; **(b)** abaxial leaf cuticle with stomata and trichome bases, Schleenhain opencast mine, Saxony, Germany, site Schleenhain 4, Böhlen Formation, Gröbers Member, earliest Oligocene (Rupelian), SPP zone 20 A/B, slide MMG PB SchleMO 11/05 from leaf SchleMO 556/2.

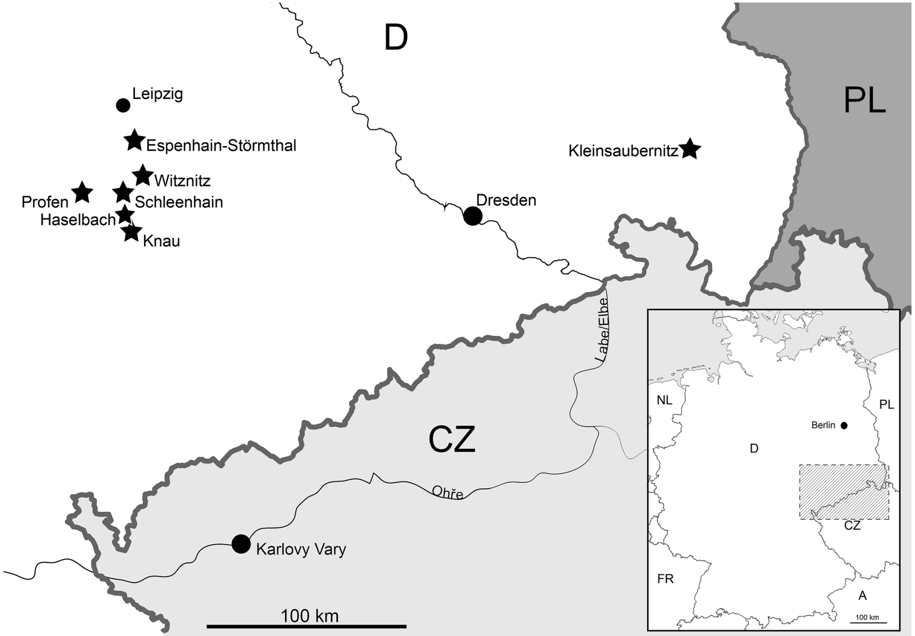

Figure 2. Sites (asterisks) of *Eotrigonobalanus furcinervis*-containing fossil taphocoenoses in central and east Germany considered in the present investigations, note: the Schleenhain and Haselbach opencast mines revealed taphocoenoses in four and two distinct lithostratigraphic positions respectively (see also stratigraphic chart in Fig. 3). Map legend: D = Germany, CZ = Czech Republic, PL = Poland, FR = France, NL = the Netherlands.

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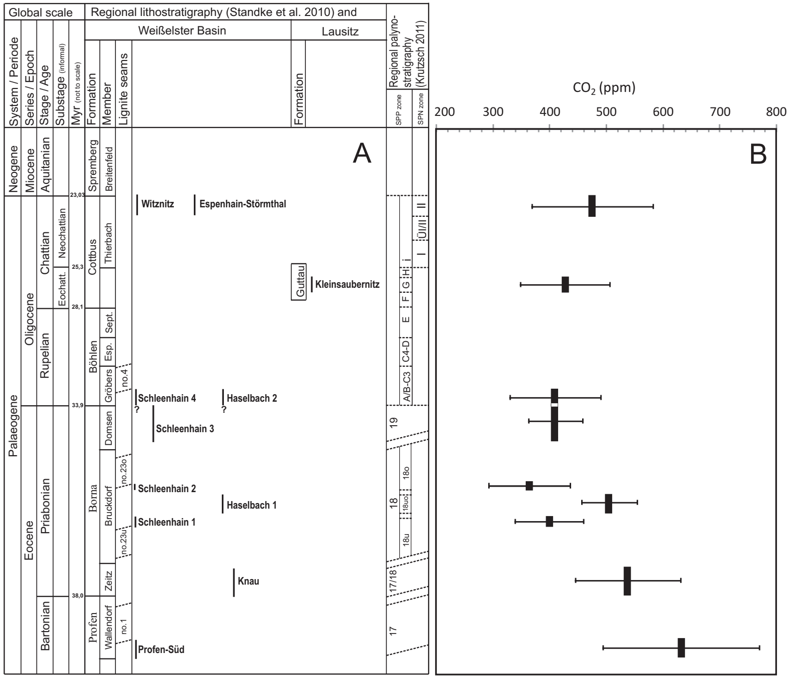

Figure 3. Stratigraphic position of the assemblages with *Eotrigonobalanus furcinervis*, regional lithostratigraphy and Krutzsch's (2011) correlation to the spore-pollen zones including his proposed correlation of spore-pollen zones to global scale (see text Sect. 2.2 for explanation and comments on dating uncertainty); black vertical bars next to assemblage names are the temporal uncertainty (based on a combination of lithostratigraphic information of the respective unit and spore-pollen zonation); bars of Schleenhain 1 and 2 are not to scale because gaps in the sediment deposition of the respective units are not equivalent to the duration of spore-pollen zones; gaps between the Eocene spore-pollen (sub-)zones illustrate gaps in the terrestrial sediment record, i.e. erosion. For horizon information see Table 1.

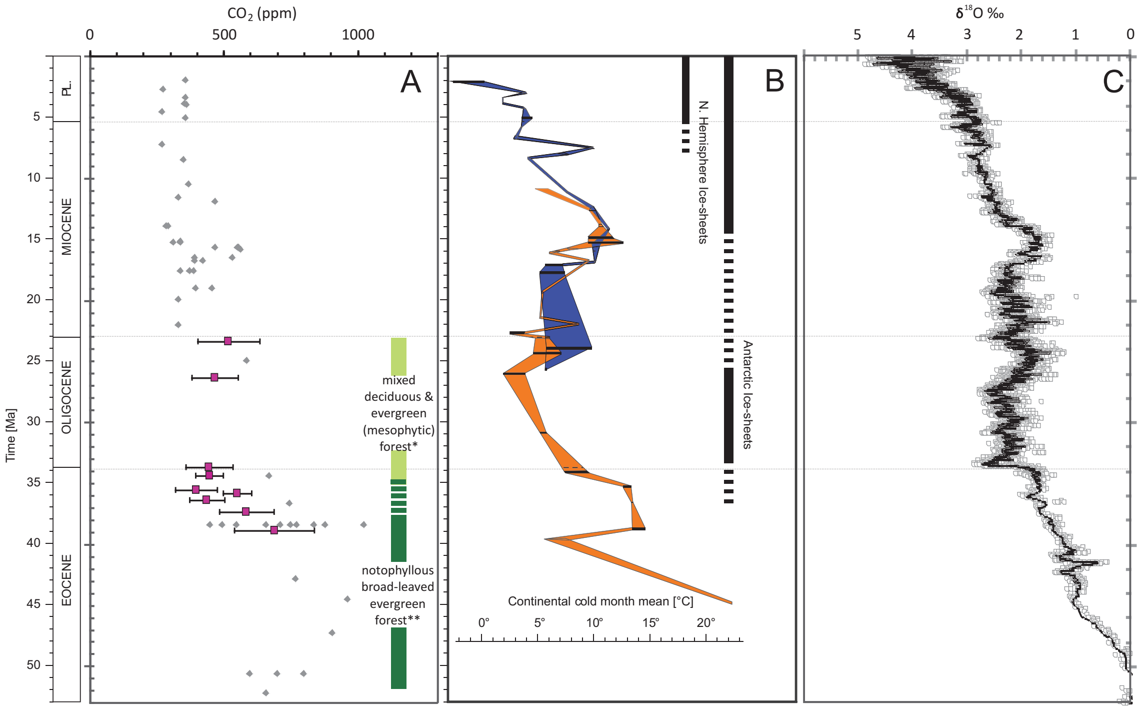

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Figure 4. CO_2, vegetation and climate trends through the Cenozoic. The most significant changes in pCO_2, forest ecosystem composition **(a)** and continental climate as tracked by terrestrial plants **(b)** take place in the late Eocene, whereas the most significant change in global temperatures as tracked by marine isotopes **(c)** takes place at the Eocene–Oliogocene boundary, indicating that the significant climate transition at the Eocene–Oligocene boundary was preceded by a gradual decrease in pCO_2 during the late Eocene. **(a)**: pCO_2 estimates from fossil stomata (this study pink with black error bars) in the context of existing stomatal proxy estimates (in grey from Beerling and Royer, 2011) in a chronostratigraphic framework. Vertical bar shows the gradual late Eocene vegetational restructuring of the dominantly evergreen forests of the Weißelster and North Bohemian basins studied here (dark green to light green), suggesting a potential causal role of pCO_2 decline in the changing ecological forest composition (* Kunzmann and Walther, 2012; ** Kvaček et al., 2014; Kunzmann et al., 2015). Note that the assigned ages for CO_2 values from this study are estimated based on the biostratigraphic controls presented in Fig. 3. Absolute ages were not available for any of the nine fossil study sites (Table 2) although clear superposition information is available throughout allowing good estimates of the temporal sequence of CO_2 estimates (see Fig. 3). **(b)**: continental temperature curve: record of continental cold month mean temperature for Central Europe during the last 45 My, redrawn from Mosbrugger et al. (2005). Horizontal bars represent coexistence intervals. Orange curve shows data from the Weißelster and Lausitz Basins, northeast Germany; blue curve shows data from the Lower Rhine Basin, northwest Germany (see Mosbrugger et al., 2005 for details). **(c)**: global climate (temperature) curve derived from stacked records of deep-sea benthic foraminiferal oxygen-isotopes: a proxy for relative changes in marine temperature in the late Eocene prior to ice build up, based on updated records from Deep Sea Drilling Project and Ocean Drilling Program sites. Raw data is smoothed by using 15-point running mean, to minimize biases introduced by uneven temporal and spatial distribution of records (data from Zachos et al., 2001, 2008; and references therein).

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