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A key for the identification of conifer stomata from N.E. China based on fluorescence microscopy

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ABSTRACT

Stomata analysis is an important tool for palaeoecological and palaeoenvironmental research. Coniferous stomata are lignified and similar in size to pollen grains. Detached from the epidermis, individual stomata can be preserved in Quaternary sediments and are often found in palynological preparations. Their identification is important for environmental and climatic interpretation. By using macrofossil remains of needle leaves and fresh needle leaves, in this study a regional identification key to the species level for coniferous stomata from Long Gang Volcanic Field (N.E. China, Jilin Province) is presented. For the microscopic investigations and the measurement of stomatal features, fluorescence techniques were applied. Three types of fluorescence filter sets using ultraviolet (UV), blue, and triple mixed excitation wavelengths were employed. To test the key and the fluorescence effect with fossil material, individual stomata from palynological slides were studied. Our results show that individual stomata have similar fluorescence to those in situ on macrofossil needles. For future stomata research, fluorescence microscopy using blue light excitation is strongly recommended.

1. Introduction

Stomata are "natural valves" for the gas exchange of plants and the pathway for water evaporation. Different ecological factors, such as light, humidity, temperature, and CO_2 concentration, can stimulate leaves to develop stomatal features to adjust for environmental change, and stomata themselves can react to those factors immediately (Willmer, 1983). Short-term increased atmospheric CO_2 concentrations can even be recorded by one single tree by means of a reduction of the stomatal frequency (Wagner et al., 1996). Based on the analysis of quantitative stomatal features, such as stomatal index or stomatal density, atmospheric CO_2 concentrations over various time spans, from recent to historical to even geological times of millions of years, can be reconstructed (e.g., Beerling et al., 1993; Royer, 2001; Wagner et al., 2004; Finsinger and Wagner-Cremer, 2009).

Unlike most angiosperm stomata, the stomata of conifers are lignified. In Quaternary lake sediments they can be preserved either as a part of leaf fragments or as detached individual stomata after the decay of the leaves. Analysis of conifer stomata has been applied in palaeoecological research such as the reconstruction of the dynamics of tree-lines (Leitner and Gajewski, 2004; Pisaric et al., 2003) and the presence of pioneer trees (Froyd, 2005; Gervais et al., 2002; Hansen, 1995; Li and Li, 2015). Conifer stomata have also been used for the reconstruction of atmospheric CO_2 concentrations (Kouwenberg et al., 2003; Doria et al., 2011; Maxbauer et al., 2014). Individual conifer stomata have a similar size as pollen grains, and can be analysed alongside pollen from the same palynological preparations (MacDonald, 2001). Their advantage for palaeoecological and palaeoenvironmental studies compared with pollen is better insight in the presence or absence of conifer species within the catchment of sedimentation sites (mostly lakes and mires). This enables an improved differentiation between local and regional signals.

A key for conifer stomata types for any study region is needed to identify individual fossil stomata from sediments. Based on fresh conifer needles as a reference, identification keys for conifer stomata from central Europe (Trautmann, 1953), North America (Hansen, 1995), and Northern Europe (Sweeney, 2004) have previously been developed. In China, stomata analysis has only recently received the attention of palynologists (Zhang et al., 2011; Shen et al., 2013; Li and Li, 2015). Except for taxonomic studies on the morphology of conifer stomata from Northwest China (Wan et al., 2007) and local palaeovegetational interpretation from the Loess Plateau (Zhang et al., 2011), stomata data from the soil surface have been used to distinguish different types of conifer forests (Shen et al., 2013).

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Here, we developed a key for stomata analysis of conifers from N.E. China, and in particular from the Long Gang Volcanic Field (LGVF, Jilin Province), which is one focus region used to study the East-Asian monsoon (Mingram et al., 2004; Schettler et al., 2006; Stebich et al., 2009, 2015; Hong et al., 2009; Wang et al., 2012; Xu et al., 2014). Based on palynological and other analyses, the regional palaeovegetation and palaeoclimate from the LGVF has already been studied in detail (Yu et al., 2008; Cui et al., 2006; Stebich et al., 2007, 2009, 2015). More studies into the local palaeovegetation and palaeoenvironment of the LGVF should be done.

The stomata used for this study are from fossil coniferous leaves, leaf remains, and fresh needle leaves from our research region and the Beijing Botanical garden. Because the investigations of stomata are usually performed alongside palynological analyses, transmitted light microscopy is the most common technique used for analysis of individual stomata. However, during our investigation, we found that stomata images under a transmitted light microscope could be ambiguous due to varying preservation conditions, in particular, for the stomata of old needles from sediment cores. By using a fluorescence technique with incident light excitation of different wavelengths, uncertainties with respect to single stomata identification could be reduced. Our local investigation may act as a case study of how fluorescence microscopy can be successfully applied to species-level identification of conifer stomata in palynological preparations.

2. Materials and methods

2.1. Sampling

Currently, the Long Gang Volcanic Field is covered with temperate deciduous broad-leaved forests dominated by *Tilia, Acer, Ulmus propinqua, Fraxinus mandshurica, Juglans mandshurica*, and temperate needle-leaved and broad-leaved mixed forests, which are comprised of *Pinus koraiensis*, *Abies holophylla*, *Quercus mongolica*, *Carpinus*, *Betula,* and other broadleaves mentioned above (Editorial Board of Vegetation Map of China, Chinese Academy of Science, 2001). Although there are other pine species, such as *Pinus densiflora*, *Pinus pumila*, *Pinus sylvestris*, and *Pinus tabulaeformis* distributed in Jilin Province, these do not spread into the LGVF (Zhou, 2010). The fresh needles of *Abies holophylla, Abies nephrolepis, Larix olgensis, Picea jezoensis*, *Picea koraiensis*, and *Pinus koraiensis,* which are currently spread widely in the LGVF, were collected at different sites from Jilin Province (N.E. China) during a field trip in October 2010. The needles of *Taxus cuspidata* were collected in 2013 from the Beijing Botanical Garden of the Institute of Botany, Chinese Academy of Sciences.

Fossil conifer needles have been sampled from the sediment cores of Sihailongwan Maar Lake (one of the nine maar- and crater lakes of the LGVF). The cores were collected in 2001 during a joint Chinese-German drilling campaign, and the sediments are shown to be varved (Mingram et al., 2004). The cores are preserved in cool storage at the German Research Centre for Geosciences (GFZ) in Potsdam (Germany).

2.2. Stomata preparation and microscopic technique

Concerning modern leaves, there are five basic methods to prepare stomata slides and perform the stomata analysis. The first method is to simply peel off the epidermis after cutting the needle leaves into strips (Weyers and Travis, 1981; Weyers and Meidner, 1990). The second method uses silicone rubber and nail varnish to obtain stomata "negatives" or "impressions" (Weyers and Johansen, 1985). Sometimes, using only nail varnish to obtain the leaf surface impression is sufficient. The third method is to use different chemicals to remove the mesophyll between the upper and lower epidermis and subsequent staining with biological dyes such as safranin. Even the epidermis may be removed, so that stomatal features can be studied from cuticle preparations (Kouwenberg et al., 2003). All three methods mentioned above use standard transmitted light microscopy to investigate the stomata features such as the length and the width of the upper woody lamellae, the length and width of the stem, and the angle between the upper woody lamellae and the stem. The scanning electron microscope is the fourth powerful tool for stomata research (e.g., Westerkamp and Demmelmeyer, 1997), but requires sophisticated and costly equipment. For some morphological studies of stomata, chemical treatment is also necessary if the leaves are covered by epicuticular wax (Hu et al., 2007), or in some cases, a mechanical artifice has to be used to remove the trichome layer. The fifth method is to use the fluorescence technique. Using fluorescence microscopy, the stomata or various epidermal appendices, such as trichomes, can be easily measured. However, this method can only be applied for those plants whose guard cells exhibit auto-fluorescence (Karabourniotis et al., 2001).

For stomata studies on fossil leaves, not all of the methods developed for modern leaves are suitable due to the fragile nature of fossils. Generally, the observation is performed under standard transmitted light microscopes or a scanning electron microscope (Dilcher, 1974; Kerp, 1990). Because of the presence of a highly aliphatic and resistant biopolymer, most observations in the pre-Quaternary palaeobotanical record are based on cuticle preparations. Also a fluorescence technique has been already used (Kerp, 1990). This technique identifies the cuticular characters for the taxonomic identification of plant macrofossils (e.g., Hu, 2007) or can be used for stomatal index data collection (Kouwenberg et al., 2003; Steinthorsdottir et al., 2013). There is an advantage of using the fluorescence technique for fossil research because the fossil material does not need to be chemically treated and will not be damaged (Kerp, 1990).

In our study, fluorescence microscopy is applied to investigate the morphological features of stomata from coniferous needle leaves. Aside from two widely used broad-band filter sets with ultra-violet and blue excitation filters, a mixed triple-wavelength filter set was used for the first time here. We used an *Olympus BX53* microscope with three types of fluorescence filter cubes (Olympus UV filter cube with a 330–385 nm excitation band; a blue filter cube with a 460–495 nm excitation band; and a triple mixed filter cube with 385–400 nm, 475–492.5 nm and 545–565 nm excitation bands; each cube contains a matching set of an excitation filter, a dichromatic mirror and an emission filter) to test for the best stomata auto-fluorescence light emission characteristics. The measurements were made using the Olympus software package "CellSens Dimension".

2.2.1. Preparation of stomata from fresh coniferous needle leaves

Fresh needles of *Abies holophylla*, *A. nephrolepis*, *Larix olgensis*, *Picea jezoensis*, *Picea koraiensis*, *Pinus koraiensis*, and *Taxus cuspidata* were treated with a 2.8% NaClO solution for several hours to several days until the mesophyll could be removed, when the whole needle turns white or transparent. After the upper and lower epidermises were separated with a small knife and a steel needle, the remains of the mesophyll were gently brushed away with a soft brush. Thereafter, the lower epidermis has been mounted with glycerin on a standard glass slide (76 mm × 26 mm) and covered by standard cover glass (0.17 mm thick).

2.2.2. Preparation of the stomata from fossil conifer needle leaves

After careful examination of the split core sediment surfaces and the thin section photos of the 26.6 m long core, numerous sediment layers with enrichments of plant macrofossils were allocated and sampled. Sediment slices were cut from individual, mm- to cm-thick macrofossil layers with a razor blade and treated with 30% H_2O_2 in a glass beaker up to 5 min until the sediments were disaggregated. The disaggregated sediments were sieved very gently with a mesh diameter of 250 μm using a soft brush and tap water through