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A new method to prepare clean cuticular membrane from fossil leaves with thin and fragile cuticles

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Leaf cuticle analysis has long been a powerful tool for fossil plant identification, systematics, and palaeoclimatological reconstruction. In recent decades the application of stomatal frequency data that are relied on precise calculation of stomata on plant fossil cuticles to reconstruct ancient atmospheric CO_2 concentration made the preparation of cuticular membrane with sufficient size a critical technique in palaeoclimatological research. However, for plants with originally thin and fragile cuticles, e.g., most deciduous plants, conventional techniques sometimes fail to obtain cuticular membranes with sufficient size, or sometimes unable to recover any. This has largely hampered the usage of fossil cuticle analysis in palaeobotanical and palaeoclimatological research. Here, we describe a new method using clear nail polish as a medium to "strengthen" the originally thin and fragile cuticles prior to maceration procedures. We demonstrate the method by using middle Eocene *Metasequoia* fossils that were notorious for the difficulty of recovering large-sized clean cuticular membranes due to their thin and fragile nature. *Metasequoia*, with well-documented and widely-distributed fossil records since the Late Cretaceous and with a living representative, *M. glyptostroboides*, as a comparative reference, has been widely used as a model genus for the study of evolution of plants, palaeoclimatological reconstruction, and plant adaptation to climate changes. But its deciduous habit produces thin cuticles and makes the preparation of clean cuticular membranes a tedious process. The new method successfully allows us to recover its delicate cuticular membranes with sufficient sizes for SEM observation and stomatal frequency analysis.

fossil leaf cuticle, *Metasequoia*, new method, thin and fragile cuticle, SEM observation, stomatal frequency, CO_2

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Terrestrial plant leaves are covered by cuticles which are composed of resistant chemicals of cutin and sometimes cutan [1–4]. Due to this layer of protection, plant leaf fossils are resistant to post-depositional decay, and sometimes are the only organs of ancient plants found in terrestrial sediments. As cuticles are tightly adhered to epidermal cells, many epidermal information including cell type (e.g., guard cells, subsidiary cells, ordinary epidermal cells), size, shape, orientation, and arrangement, can be obtained from cuticular membranes. Furthermore, as the barrier between the interior of plants and its external environment, epidermal cells and their cuticle coverage record unique information regarding plant's physiological responses to climate changes. All these make cuticle analysis one of the most widely used techniques in palaeobotanical and palaeoclimatological research. In addition, since the demonstration of the correlation between plant leaf stomata and the ambient CO_2 concentration [5], the past decades have witnessed a remarkable

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increase of the application of stomatal frequency (SF) expressed as stomatal density (SD), stomatal index (SI), and stomatal number per (conifer needle) length (SNL) [6–9] for the quantitative reconstruction of ancient atmospheric CO_2 concentration [10–13]. The challenge to apply this method has been to obtain fossil cuticular membranes with sufficient size to yield quantitative biological information with statistical significance.

However, due to diagenetic alteration, and more importantly, due to the originally thin and fragile nature of cuticles for some plants (e.g., most deciduous plants, both angiosperms and gymnosperms), it is often difficult to obtain clean cuticular membranes, particularly membranes of the lower epidermis (or epidermis with stomata), with sizes large enough for stomatal frequency calculation.

In this paper, we describe a new preparation method that was derived from two conventional procedures of obtaining "clean cuticle" and "transfer of cuticle" (see below) by using clear (colorless) nail polish (nitrocellulose) as a cuticle "strengthening" medium. This new approach allows the originally thin and fragile fossil cuticles to be fixed prior to the maceration process for the recovery of clean cuticular membranes with sufficient sizes.

We use leaf fossils of *Metasequoia* to demonstrate this new method, and the selection has a dual purpose. First, although *Metasequoia* is a conifer, its deciduous habit makes its cuticles, particularly the lower cuticles, much thinner and more fragile than those of other evergreen conifers, making it difficult to prepare large pieces of clean cuticular membranes from fossil leaves. Second, with a continuous and widely distributed fossil record dated back to the Late Cretaceous in the Northern Hemisphere [14–16], and even more importantly, with a representative species (*M. glyptostroboides* Hu et Cheng) still living in its native land in Southwest-central China [17, 18], *Metasequoia* has long been used as an effective paleoclimatic proxy for the past 100 million years of the Earth history [19–24].

1 Materials and methods

1.1 Materials

Leaf fossils of *Metasequoia occidentalis* (Newberry) Chaney used for this case study were collected from the ~45 million years old middle Eocene Buchanan Lake Formation, Axel Heiberg Island, Nunavut, Canada (fossil location refers to Figure 1 of ref. [25] and fossil identification follows Liu and Basinger [26].) The specimens were well preserved and have been reported to yield *in situ* chloroplasts and other cellular components and even *in situ* liable biomolecules [25–29]. However, even though the exceptional preservation was described as "mummified," due to the originally thin and fragile nature of cuticles, clean cuticular membranes of certain size, particularly of the lower epidermis, have never been successfully obtained previously from these fossils.

1.2 Methods

1.2.1 Pretreatment for dissolving adhering sedimentary matrix

Any sedimentary matrix adhering to the fossils should be dissolved by the treatment of 1) 10%–25% hydrochloric acid (HCl) for 2 hours (h), 2) 40% hydrofluoric acid (HF) for 12 h, and 3) 10%–25% HCl for at least 1 h. The treated fossil leaves are then rinsed in water for 3 times with each time for 10 minutes (min). The middle portion of the leaf is then cut off and air-dried (generally overnight) for the second procedure.

1.2.2 Applying clear nail polish

A thin layer of clear nail polish is applied onto the lower epidermal surface of the air-dried middle portion of the matrix-free fossil leaf material, but leaving the margin free of the painted chemicals. Generally the upper cuticle is thicker, and thus there is no need to be "strengthened." The specimens are then air-dried for about 2 days (d), until the nail polish becomes a thin film adhering tightly to the epidermal surface.

1.2.3 Clean cuticular membrane maceration

Maceration methods for obtaining clean cuticular membranes have been systematically summarized by Dilcher [30] and Kerp [31]. For originally thin and fragile cuticles, mild maceration reagents are always recommended and special care is required during the process to assure the best results as the reaction time may vary from seconds to hours.

For our Eocene *Metasequoia occidentalis* specimens, after repeated testing, we have found out that the following maceration procedures produced the most satisfactory result:

The whole specimen (with the thin clear nail polish film attached) is macerated with a weak Schulze's reagent: 65% nitric acid (HNO_3) with very few potassium chlorate ($KClO_3$) crystals, similar to the "dry Schulze's reagent" described in ref. [31]. Once the color turns lighter and small bubbles can be observed between the upper and lower epidermis, the reaction should be stopped immediately by carefully rinsing the specimen with water for several times. Then the upper and lower epidermis can be separated by using a thin dissecting needle or a hand-made single hair brush (a special needle with a rigid human hair glued at the tip). Remnants of mesophyll and vascular bundle can be partly removed.

The separated epidermis (the lower one with the clear nail polish film attached) are then treated by 3%–5% sodium hypochlorite (NaClO) solution to disintegrate the remnants of mesophyll, vascular bundle, hypodermal layer, and epidermal cell walls into amorphous material. This may

take 10 min–2 h. If there is no reaction up to 2 h, a treatment of 5%–10% aqueous ammonia ($NH_3 \cdot H_2O$) solution for 1–3 min can completely remove any remaining remnants attached to the internal surface of the cuticle. Clean cuticular membrane can be obtained after thoroughly rinsing with water. The clean cuticular membrane of the lower epidermis is still adhered to the thin nail polish film.

Instead of using NaClO solution, remnants of mesophyll, vascular bundle, hypodermal layer, and epidermal cell walls attached to the separated upper and lower epidermis can also be removed by even milder reagents such as 30% hydrogen peroxide (H_2O_2). This process may last for 2 d to even one month depending upon fossil preservation, and the long time treatment does not cause any curling or damaging to the cuticular membrane.

The cleaned cuticular membranes can now be transferred to a scanning electron microscope (SEM) stub with the internal surface of the cuticle facing upwards for SEM observation.

2 Results and discussion

For the first time, this method enables us to obtain large-sized pieces of clean cuticular membrane of the lower epidermis of middle Eocene *Metasequoia* leaves collected from the Buchanan Lake Formation, Axel Heiberg Island, Nunavut, Canada (Figure 1). These pieces are large enough for most purposes of cuticle analysis, e.g., the observation on type, size, shape, orientation, and arrangement of epidermal cells, the observation on fine sculpture of the internal surface of cuticle, and the precise calculation of stomatal frequency. While detailed description of these morphological characters and the usage of stomatal data will be presented elsewhere [32], the information brought about by this new method has the following immediate implication:

The large-sized clean cuticular membranes obtained using our new method allow us to precisely calculate the number of stomata vs. clearly defined epidermis cells—a condition that is required for stomatal frequency calculation. For example, on the clean cuticular membrane shown in Figure 1, SD was calculated as 298.98 mm^{-2}, SI as 10.85, and SNL as 128.14 mm^{-1}. It is worth mentioning that the "anticlinal walls" (term adopted from epidermal cells for the internal intrusion of cuticle between two epidermal cells) sometimes are too thin or too low to be readily recognized even under SEM observation (e.g., Figure 1(b)). Until the application of this new method that offered refined image of these anticlinal walls, the number of cells could not be reliably determined for some fossil *Metasequoia*. Thus, our results suggest that *Metasequoia* stomatal frequency data counted through conventional light microscope (LM) observation for the purpose of reconstructing ancient CO_2 concentration should be treated with caution.

As one of the most widely used palaeobotanical techniques, cuticle analysis is largely based upon methods of cuticle preparation. The following four categories of methods have been described [30, 31, 33, 34]. 1) Method to prepare "clean cuticle" (or "clean cuticular membrane," in ref. [30] under "whole mounts of the cuticle" method). This includes mainly the oxidation procedures to clean other organic remains and to bleach the cuticular membrane by maceration. Schulze's reagent (HNO_3 + $KClO_3$), chromium trioxide (CrO_3), NaClO, and H_2O_2 are commonly used reagents [30, 31, 34]. 2) Method to obtain "transfer of cuticle". For fossil leaf compression with thin and fragile cuticle which tends to disintegrate completely during maceration, we may try to "transfer" the cuticle to a thin film to make them "glued" or "fixed" together by coating the fossil cuticle with a liquid such as collodion dissolved in ether or in EtOH:ether (3:1, v/v), a clear lacquer, a clear celluloid tape (e.g., Scotch Brand #853 manufactured by 3M), and acetone-based clear nail varnish (polish) (nitrocellulose) followed by a celloidin-based glue [30]. This method was considered less satisfactory [31, 33] until recently polyester overlays, a common overlaminates for laboratory glassware labels, was applied to make thin films [33], which enhanced the usage of this method. 3) Method to make "replica of cuticle." Acetate film, silicone rubber, and rubber latex have been reported to be used for making replicas of fossil cuticles. However, as fossil cuticle is generally not well enough preserved, this method has limited approaches [30]. 4) Direct observation. In rare and ideal cases when leaf compressions are in good preservation cuticular characters might be directly observed without any treatments. For example, stomatal counts can sometimes performed under an epifluorescence microscope directly on the leaf specimen when its epidermis displaying stomata facing upwards in the rock matrix [33].

Our method combines the first two methods. We first use nail polish to "strengthen" the thin and fragile cuticle, and then macerate the material in reagent(s) to obtain clean cuticular membrane which is still adhered to the thin clear nail polish film. Commercial products of colorless nail polish had long been used in cuticle preparation, both in the "transfer of cuticle" method and the "replica of cuticle" method [30, 31, 33], as well as in cuticle preservation [35]. But compared with other material, the results by using nail polish have been generally reported as unsatisfactory [30, 31, 33]. Kerp [31] reported that nail polish treated material cannot be further macerated with Schulze's reagent. However, our experiment results have demonstrated that clear nail polish not only can work as a very efficient medium to "strengthen" the originally thin and fragile cuticle, but also steadily accompany the cuticle to endure further maceration treatment with Schulze's reagent to obtain large sized cuticular membranes. Moreover, the nail polish film can also prevent cuticles from curling and shrinking which generally occur to thin cuticles.

The new method has two limitations due to the applica-

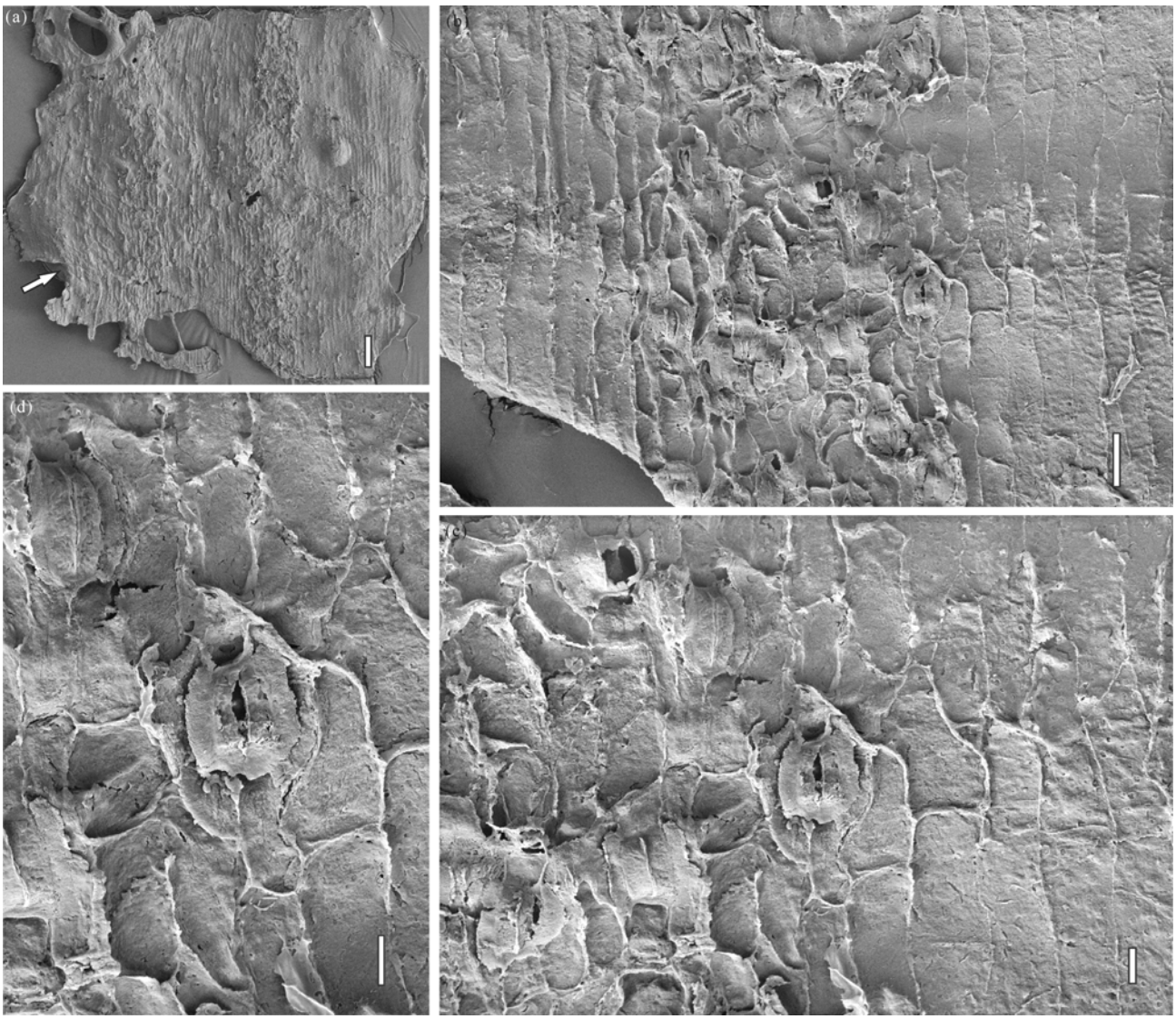

Figure 1 SEM photographs of internal surface of lower cuticle of *Metasequoia occidentalis* leaf fossils from the middle Eocene Buchanan Lake Formation, Axel Heiberg Island, Nunavut, Canada. Both stomatal zone (middle) and non-stomatal zones are shown. (a) Overview of the whole clean cuticular membrane with nail polish film partly seen (arrow). Scale bar = 100 μm. (b) Lower part of (a) enlarged, showing the whole width of the stomatal zone (middle). Note the thin anticlinal walls, particularly in the stomatal zone. Scale bar = 30 μm. (c) Middle-right part of (b) enlarged, showing internal fine sculpture of both stomatal zone (left) and non-stomatal zone (right). Scale bar = 10 μm. (d) The stoma at the center of (c) enlarged, showing details of the internal structure of stomata and ordinary epidermal cells. The guard cells bear prominent frill-like flanges. Scale bar = 10 μm.

tion of nail polish: 1) the outer surface morphology of the obtained cuticle is covered by the nail polish film, thus cannot be further observed, and 2) the adhering nail polish film does not allow the cuticle to be stained and mounted as a glass microscope slide for LM observation. However, as the outer surface morphology can be readily obtained directly on untreated leaf fossil specimen (e.g., under SEM with the lower epidermis facing upwards), and SEM can generally provide better resolution than LM, the advantages of this method far outweigh these two limitations. The newly innovated ESEM (Environmental Scanning Electron Microscope) which allows fresh (even alive) specimens to be observed directly without coating and vacuuming would make SEM observation even more convenient.

3 Conclusion

We describe a new cuticle analysis method that is effective for recovering large-sized clean cuticular membranes from originally thin and fragile plant leaf fossils. Built upon and developed from two previously separated procedures, the new method applies clear nail polish as a medium to strengthen delicate cuticles before maceration procedures. Using this new method, we recovered large-sized clean cuticular membranes from ~45 million years old middle Eo-

cene *Metasequoia* fossil leaves that were previously failed using traditional methods. The obtained membranes allowed us to obtain more precise stomatal frequency calculation for palaeoclimatological reconstruction. We believe that this new method can be universally applied to other leaf fossils with originally thin and fragile cuticles, thus opening the door to observe more micro-morphological characters as well as stomatal frequency on delicate fossil plant cuticles.

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