Review of Palaeobotany and Palynology 232 (2016) 22–39

Contents lists available at ScienceDirect

Review of Palaeobotany and Palynology

journal homepage: www.elsevier.com/locate/revpalbo

Insights into the leaf cuticle fine structure of *Ginkgoites skottsbergii* Lundblad from the Albian of Patagonia and its relationship within Ginkgoaceae

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ARTICLE INFO

Article history:
Received 21 November 2015
Accepted 9 May 2016
Available online 25 May 2016

Keywords:
Ginkgoites skottsbergii
Leaf cuticle
Ultrastructure
Ginkgoaceae
Albian
Argentina

ABSTRACT

New specimens of *Ginkgoites skottsbergii* Lundblad collected from the Albian Kachaike Formation at the type locality in Santa Cruz Province, Argentina are characterized with additional scanning and transmission electron microscopic observations and EDS elemental analysis. The micromorphology of the amphistomatic and 12-segmented leaf of *G. skottsbergii* shows a smooth lamina with straight and pitted anticlinal and slightly granulate periclinal walls, abundant epicuticular wax on the petiole surface, and monocyclic to partially dicyclic stomatal apparatuses with between six and eight papillate to non-papillate, finely striated subsidiary cells and guard cells with smooth walls. Palaeoenvironmental considerations suggest that *G. skottsbergii* was a mesophytic plant. The TEM studies of ordinary epidermal cell cuticles and subsidiary and guard cell cuticles reveal the general ultrastructural features of Ginkgoaceae. Using confidence intervals based on 30 statistical measurements and the Mann–Whitney test for five measurements of EDS ratios of the elements, 33 ultrastructural characters were identified that enabled discussion at different taxonomic levels. Comparisons with the previously studied *Ginkgoites ticoensis* Archangelsky reveal that 14 of these characteristics could be species features that reinforce the identity of *G. skottsbergii*, and 19 could be genus features. EDS analysis and comparisons with *G. ticoensis* yielded five ratios using Cl, N, K, S, and Ca, revealing seven ratios of potential species interest and eight ratios at the genus level among the three zones evaluated in the leaf cuticle of each species. A three-dimensional reconstruction of the cuticle and an ultrastructural identification key for each of the three types of cuticle are also provided, i.e., from the ordinary epidermal cell and the subsidiary and guard cells of the stomatal apparatus. Fruitful statistical comparisons with two previously studied ginkgos allow discussion of the relationships within the Ginkgoaceae with respect to 18 features, six of them being potentially significant at the genus level and four at the family level.

1. Introduction

During the Mesozoic, the ginkgophytes were one of the most diversified and widespread groups of gymnosperms; they began to radically decline in the Cenozoic and are currently restricted to East Asia (Crane, 2013). In Argentina, the fossil record of the group can be traced back to the late Palaeozoic (Frenguelli, 1946) and represented by several taxa, although the diversity increased during the Mesozoic. This richness is particularly observed in the Cretaceous of southern Patagonia, where several vegetative and reproductive remains have been found. Among the first records, three species of *Ginkgoites* Seward were well-established in that area. *Ginkgoites tigrensis* Archangelsky and *Ginkgoites ticoensis* Archangelsky were part of the well-known Baqueró Flora of the Aptian age (Archangelsky, 1965, 2003), whereas *Ginkgoites skottsbergii* Lundblad is part of the Lake San Martín plant community of Albian age, described first by Halle (1913) and then by Passalia (2007a, 2007b). Like most of the fossil plants recovered from these two fossiliferous sites, leaves of the three *Ginkgoites* species consist of compressions with well-preserved cuticle features.

Studies of the leaf cuticles of *G. tigrensis* and *G. ticoensis* based on observations with light, scanning and transmission electron microscopy have clearly shown that the two taxa are quite distinct, although their leaf gross morphologies are rather similar (Del Fueyo et al., 2013; Taylor et al., 1989; Villar de Seoane, 1997).

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http://dx.doi.org/10.1016/j.revpalbo.2016.05.002
0034-6667/

The leaf of *G. skottsbergii* was originally tentatively assigned to *Baiera* cf. *australis* McCoy by Halle (1913) and was later assigned to *Ginkgoites australis* by Florin (1936, p. 106). Halle based his description on the foliar gross morphology alone, while Florin simply quoted the Patagonian material in the synonymy list with no illustration or comment.

During the course of the cuticular study of the Ginkgoales from the Baqueró Flora, Archangelsky (1965, p. 125) reinvestigated the original material described by Halle at the Swedish Museum of Natural History and recovered several leaf cuticle fragments, which allowed him to confirm Florin's opinion that this ginkgoalean fossil is better included in the genus *Ginkgoites* instead of *Baiera* F. Braun.

However, in his analysis of the Victoria Flora, Douglas (1969) examined the gross morphology and foliar cuticle of new specimens of *Ginkgoites australis* and found that these Australian leaves were quite distinct from Halle's Patagonian leaves. Later, when Lundblad (1971) restudied Halle's original material, she confirmed Douglas's assumptions and assigned it to a new species, *G. skottsbergii*.

At present, the foliar cuticle of *G. skottsbergii* has been solely analysed using light microscopic observations (Lundblad, 1971; Del Fueyo et al., 2006; Passalia, 2007a). Therefore, the purpose of this contribution is to make a detailed study of the structure and the fine structure of the *G. skottsbergii* leaf cuticle using scanning and transmission electron microscopy based on new specimens collected at the type locality of Halle (1913). Our goal is to complete the characterization of this taxon to compare it with other *Ginkgoites* and *Ginkgo*-like species, particularly through ultrastructural characterization and to identify its correct family affinity within the Ginkgoales.

2. Materials and methods

2.1. Material and geological setting

The leaves of *G. skottsbergii* were recovered by one of the authors (GMDF) in 2002 from the Kachaike Formation in the Bajo Comisión locality (Estancia Sierra Nevada) in Santa Cruz Province (Fig. 1). The Kachaike Formation, named by Riccardi (1971), is exposed east of Lago San Martín, from the Río de los Fósiles in the north to the Estancia Kachaike in the south. According to Arbe (2002), the formation was deposited during the Kachaike–Piedra Clavada Regressive Subcycle of the Lago San Martín Cycle in the Austral Basin. The lower part of the Kachaike Formation in the Bajo Comisión locality consists of transgressive clastic deltaic sediments and a transition to continental deposits in the upper part, where the fossil leaves studied here were collected.

The presence of angiosperm pollen (Barreda and Archangelsky, 2006) and lycopsids and aquatic-fern megaspores (Villar de Seoane and Archangelsky, 2008) in the continental upper part of the Kachaike Formation suggest a late Albian age, whereas the spores and pollen from the lower half to upper part of this unit were assigned to the Albian (Archangelsky et al., 2012). However, the presence of ammonites and microplankton in the lower marine part of this unit reveals a late Aptian or early Albian age, respectively (Aguirre Urreta, 2002; Guler and Archangelsky, 2006).

2.2. Methods

The lamina and the petiole cuticles of *G. skottsbergii* were gently removed from the sediment with water and oxidized in 40% nitric acid followed by 5% ammonium hydroxide. Sodium hypochlorite was also used to clear some recalcitrant cuticles. For scanning electron microscopy (SEM), cuticles of the lamina and petioles were mounted on exposed film adhered to stubs, coated with gold–palladium and examined using a Philips XL30 TMP SEM at 15.1 kV at the Argentine Natural Sciences Museum Bernardino Rivadavia.

The samples for transmission electron microscopy (TEM) were prepared according to Lugardon's technique (1971), which was also used for fossil pollen and spores and for living plant cuticles (conifers and angiosperms; Bartiromo et al., 2012, 2013). From the selected remains suitable for fine analysis, three Epon resin blocks were made

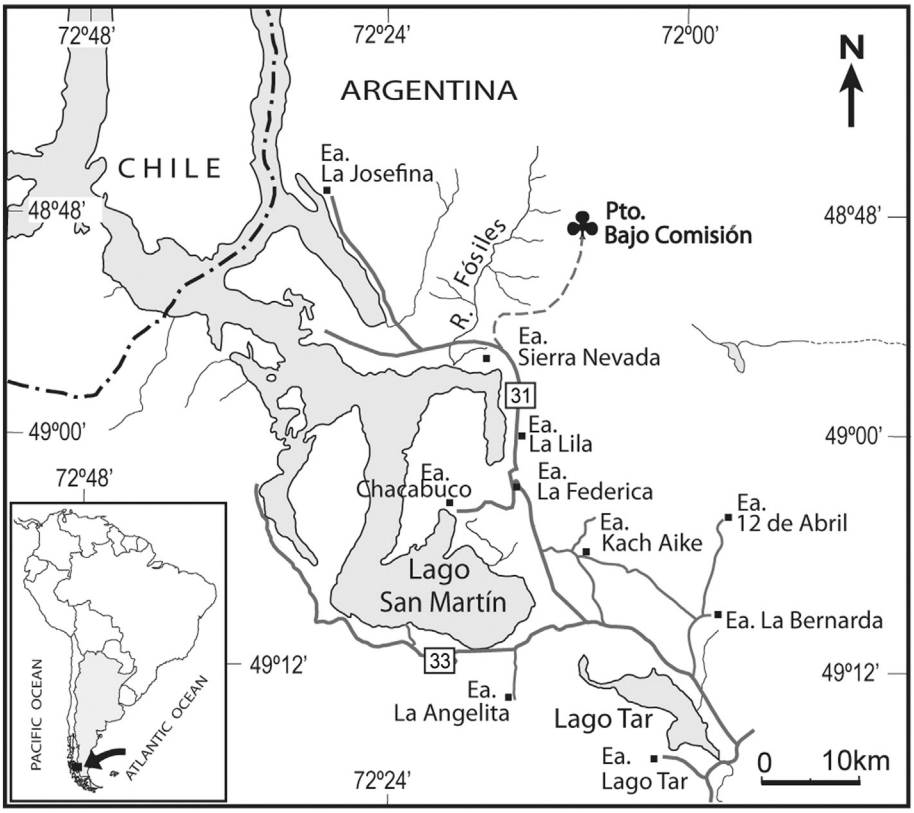


Fig. 1. Location map showing the Bajo Comisión locality at Estancia Sierra Nevada (♣), Kachaike Formation, Santa Cruz Province, Argentina.

from the leaf. From these, 110 ultrathin 60–70 nm sections were made and collected on uncoated 300-mesh copper grids (60 transverse sections, i.e., perpendicular to the leaf length; 50 longitudinal sections, i.e., parallel to the leaf length). Ultrathin sections were selected and observed and photographed with a Philips CM 120 at 80 kV at the Centre de Technologie des Microstructures (CTμ) of Lyon-1 University.

Energy-dispersive X-ray spectroscopy (EDS) was performed on the TEM sections for *G. skottsbergii* and for the previously ultrastructurally studied *G. ticoensis* using SIRIUS SD ENSOTECH and IDFIX software with an acceleration voltage 120 kV, a spot size 3, a processing time of 60 s, and a time constant of 4 microseconds. For each of the two species of *Ginkgoites* compared in this study, 10 copper 200-mesh Formvar-coated grids were used, which were devoid of uranyl acetate and lead citrate staining. Among the observed elements, Cu and Al were ignored in the results as belonging to the grid, as were Os as part of the embedding technique, Si as part of the oils used in the TEM, and C and O as major constituents of the EPON embedding resin.

The fossil specimens and samples used for SEM are deposited in the Palaeobotanical collection of the Bernardino Rivadavia Argentine Natural Sciences Museum (under acquisition numbers BA Pb 13850, 13851 and BA Pb MEB 248–251, respectively). Resin blocks and TEM negatives are stored at Lyon-1 University. The following terms and abbreviations are used in the text as well as in the tables, plates, and appendices: OEC = ordinary epidermal cell cuticle; SC and GC = subsidiary and guard cell, respectively, of the cuticle of the stomatal apparatus; CM = cuticular membrane (CP + CL); CP = cuticle proper (A = A1 + A2); A1 = outer polylamellate layer of the cuticle proper (A1U + A1L); A1U = upper part of A1; A1L = lower part of A1; A2 = inner mainly granular layer; CL = cuticular layer (B); OL = opaque lamellae of the polylamellate layer (A1); TL = translucent lamellae of the polylamellate layer (A1); OP and IP are the outer and inner parts, respectively, of the cuticle; OC and IC are the outer and inner chambers, respectively, of the stomatal apparatus.

3. Descriptions and results

3.1. Gross morphology

G. skottsbergii Lundblad is a simple, lobed, amphistomatic and petiolate leaf up to 50 mm long and 40 mm wide. The petiole is slender, up to 10 mm long and 1 mm wide, reaching 2 mm wide at the lamina base. The lamina has deep incisions up to 2.5 mm at the petiole base from which the first-order segments originate. Each of these segments shows another, shallower incision up to 6 mm at the petiole base that forms the second-order segments (Plate I, 1–3). The number of segments varies from 8 to 12; they are lanceolate to linear, 10–17 mm long and 3–4 mm wide with straight margins. The first-order segments have mostly rounded apexes, whereas the second-order segments are occasionally slightly incised (Plate I, 1–2). The petiole has two veins that dichotomise at the lamina base, where they enter and divide two or three times (Plate I, 3). Each segment shows up to eight parallel and dichotomously forked veins (Plate I, 2–3).

3.2. Epidermal structure

In *G. skottsbergii* both adaxial and abaxial epidermal layers of the segments are formed by cells with different forms, sizes and dispositions (Plate II, 1). Epidermal cells in the intercostal zones are irregularly disposed and are isodiametric to polygonal, 27–32 μm long and 16–21 μm wide (Plate II, 3). Their walls are slightly thickened (0.7 μm wide) and pitted (Plate II, 3). In the costal zones the epidermis consists of six rows of rectangular cells (41–57 μm long and 15–19 μm wide) with a straight and pitted anticlinal wall 1–2 μm thick. Externally all periclinal cell walls are finely striated (Plate II, 7), while internally they appear slightly granulate (Plate II, 2).

Stomatal apparatuses are irregularly disposed in both epidermal layers (Plate II, 1); they are monocyclic to partially dicyclic, circular, up to 50 μm in diameter, and with oval to circular pits (Plate II, 5–6). The 6–8 subsidiary cells are rectangular and up to 55 μm long and 15 μm wide. Their anticlinal flanges are straight and up to 3 μm thick, whereas their periclinal walls are internally granulate (Plate II, 4–6). The subsidiary cells show cuticular thickenings of the aperture walls and form a cuticular rim around the stomatal pit (18 μm long and 11 μm wide) (Plate II, 7) or exhibit sub-circular (7 μm high and 15 μm wide) to elongate papillae (5.5 μm high and 8 μm wide) overarching the pit (Plate II, 8–9). Some papillae show finely striated surfaces (Plate II, 8). The guard cells are sunken and reniform, up to 18 μm long and 6 μm wide; their walls are smooth and thicker (4 μm) than those of the subsidiary cells (Plate II, 4–6). The stomatal density for both leaf surfaces is 40–45 per mm^2 (Plate II, 1).

The petiole in the intercostal zones has epidermal cells that are similar in shape and size to those of the lamina, although the anticlinal walls are thicker (2.5 μm). Moreover, the epidermal cells in the costal zones are also rectangular, but narrower (34–44 μm long and 12.5–15 μm wide) and disposed along the major axis of the petiole (Plate III, 1–2). In contrast to the lamina, the surface of the petiole appears to be covered by abundant epicuticular wax (Plate III, 3).

The stomatal apparatuses are of the same type and form as those observed in the lamina, but are larger (78 μm in diameter) and many fewer. The stomatal density is 2–4 per mm^2 (Plate III, 1). Because the guard cells are partially preserved, they are difficult to measure (Plate III, 9–10). The subsidiary cells exhibit cuticular thickenings of the aperture walls, forming an oval cuticular rim around the pit (Plate III, 4, 8) or have a variable number of papillae (3–6) overarching the mouth of the stomatal apparatus (Plate III, 5–7). Externally, all subsidiary cells show a striated surface (Plate III, 4–7).

3.3. Cuticular ultrastructure

The cuticular membranes are of three types: ordinary epidermal cells (Plate IV) and subsidiary (Plates V, VI) and guard (Plate VII) cells of the stomatal apparatuses. They consist of an outer cuticle proper [A = outer polylamellate layer A1, only present in the ordinary epidermal cell cuticle (A1U in the upper part with straight translucent lamellae quite condensed in number and arranged parallel to the outer surface of the cuticle, A1L in the lower part with less condensed and rather disrupted and translucent lamellae arranged parallel or transverse to the outer surface), and granular inner layer A2 with various concentrations of elements] and an innermost fibrillar and reticular cuticular layer CL (= B1). The good preservation of the material revealed the variability at this ultrastructural level, particularly in the A2 and B1 layers (Plate IV 3–5). Statistical measurements were performed on each type of cuticle (Table 1, Appendix A in the online version of this paper). Ten characteristics were considered, making a total of 18 for the three types of cuticle: the total cuticular membrane thickness (CM), the five cuticle proper (A) thickness features, the thickness of the B layer (=B1), the thicknesses of the opaque and translucent lamellae of the A1 layer, and the number of translucent lamellae.

Ordinary epidermal cuticles (Plate IV, Table 1) have a medium total thickness (mean, 2.07 μm), while the subsidiary cell cuticle (Plates V–VI) is the thickest (mean, 2.89 μm) and the guard cell cuticle (Plate VII) is the thinnest (mean, 0.47 μm). The ordinary epidermal cell cuticle has the highest % of cuticle proper A (70.5%) and the lowest % of cuticular layer B (29.5%); conversely, the subsidiary and guard cells have a lower % of cuticle proper A (65.1 and 31.9%, respectively) and the guard cell cuticle has the highest % of cuticular layer B (68.1%). At a large magnification of the cuticle proper A, the A1 layer is only present in the ordinary epidermal cell cuticle (Plate IV, 9–13), with the two sublayers A1U and A1L quite equivalent in thickness (means of 0.025 and 0.024 μm, respectively). Their percentages vary

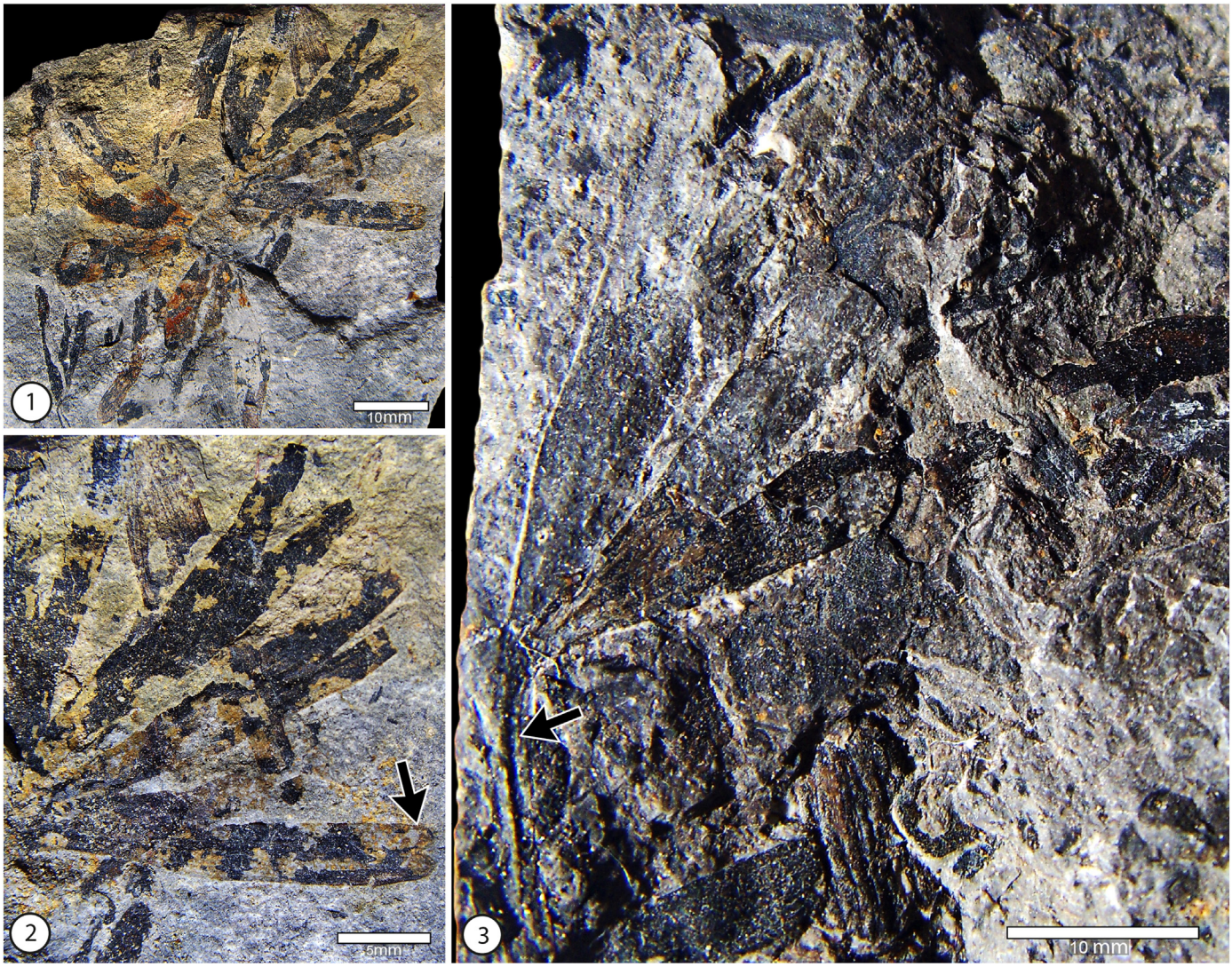


Plate I. *Ginkgoites skottsbergii* Lundblad. Leaf morphology under stereo microscope.

1. Leaf gross morphology showing numerous leaflets. BA Pb 13851.
2. Detail of leaflets. Note incised (arrow) segment apex. BA Pb 13851.
3. Detail of leaflets and petiole (arrow) in an incomplete leaf. BA Pb 13850.

in the same range. The opaque lamellae of the A1 layer are about three times thicker than the translucent lamellae (means of 13.08 versus 4.83 nm, respectively).

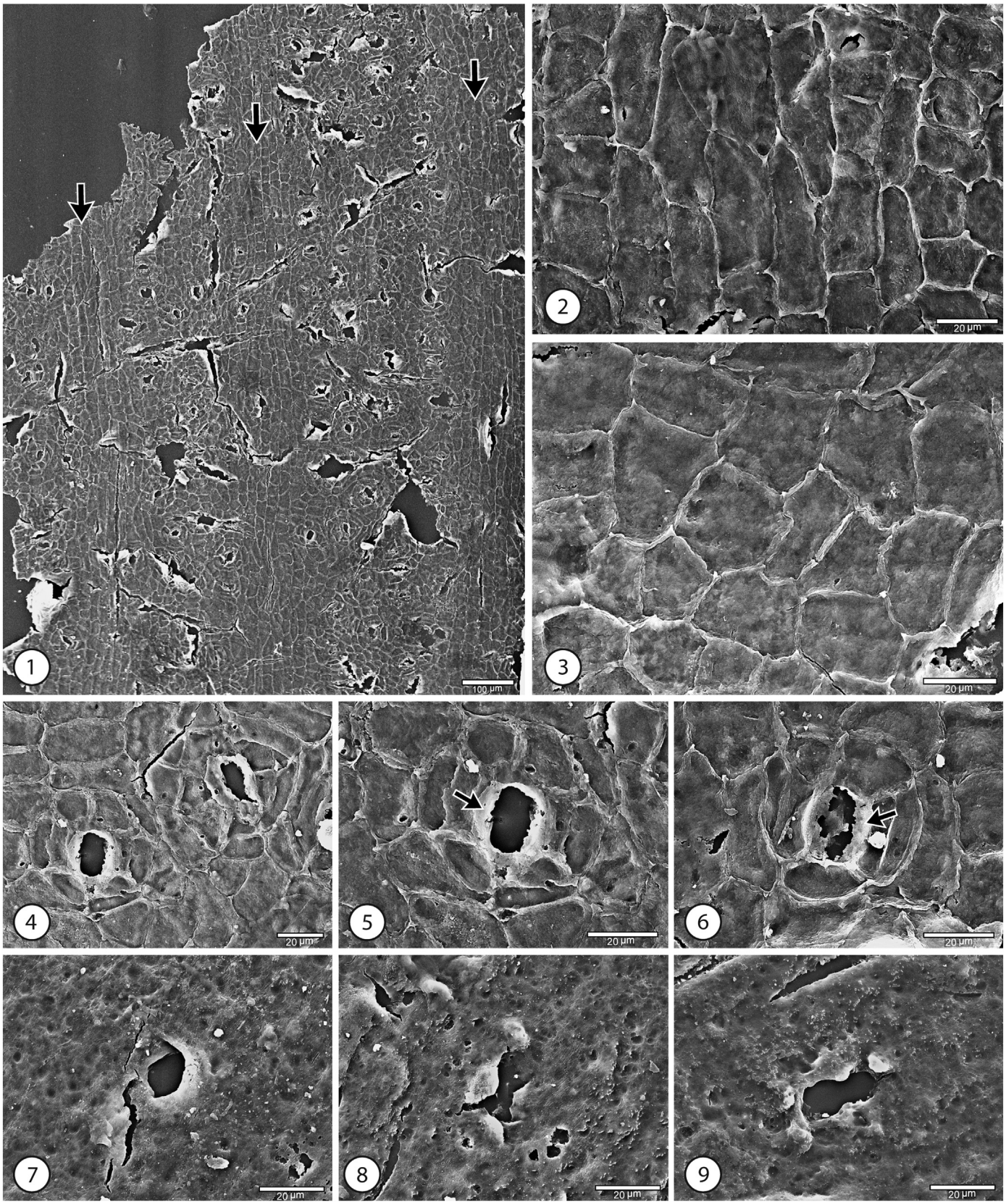
3.4. EDS elemental analysis of the ordinary epidermal cell cuticle

When analysing the cuticle with EDS the elemental measurements are added to the resin within the cuticle, although this introduces a degree of error due to the embedding resin. Hence, to provide comparable values for the cuticles, among the 10 potential ratios between Cl, N, K, S, and Ca, only five ratios (Cl/N, K/S, N/Ca, S/Ca, K/Ca) with homogeneous resin values (with insignificant differences in the Mann–Whitney test, indicated as 'no' in Table 3) are selected here, though producing similar eventual errors due to the elements in the resin (see also Table 2; Fig. 2; Appendices B, C, D, E in the online version of this paper). Moreover, it must be noted that in the two resins of each

Plate II. *Ginkgoites skottsbergii* Lundblad. Leaflet epidermis under scanning electron microscope. 1–8. BA Pb MEB 249. 9. BA Pb MEB 248.

1–6. Epidermis inner surface.
1. Epidermis in general view. Note stomatal apparatuses randomly placed in intercostal zones. Arrows indicate costal zones.
2. Detail of elongate-rectangular cells in costal zones.
3. Isodiametric-polygonal cells in intercostal zones.
4–6. Stomatal apparatuses
5. Two stomatal apparatuses with subsidiary cells in contact.
5–6. Detail of partially dicyclic stomatal apparatuses. Arrows indicate guard cells.
7–9. Stomatal apparatuses outer surface.
7. Detail of non-papillate subsidiary cells.
8–9. Detail of papillate subsidiary cells. Note ordinary epidermal cells are non-papillate.

taxon, the majority of these elements have a much lower presence than in the cuticle, although the much higher percentages of these last elements in the cuticle are really significant. Added to the confidence intervals, all of these cuticle values were determined to be significantly different or not using the Mann–Whitney test for the five measurements.



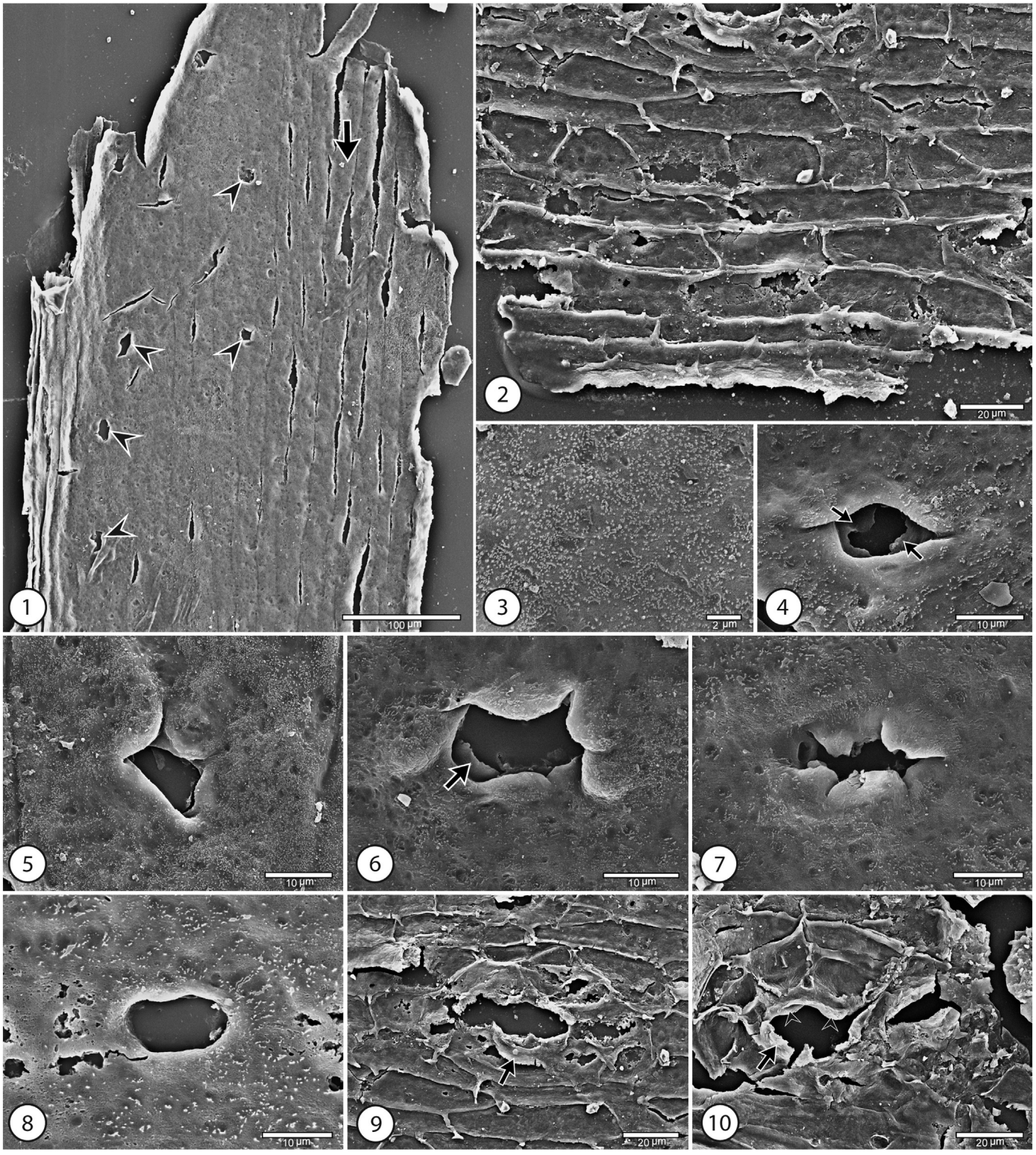


Plate III. *Ginkgoites skottsbergii* Lundblad. Petiole epidermis under scanning electron microscope.

1. Epidermis outer surface in general view. Note scarce stomatal apparatuses randomly placed (arrowhead) and rectangular cells in costal zones (arrows). BA Pb MEB 250.
2. Detail of elongate-rectangular cells inner surface. BA Pb MEB 251.
3. Detail of epicuticular wax outer surface. BA Pb MEB 250.
4–8. Stomatal apparatuses outer surface. Note variable number of papillae on subsidiary cells. Arrows indicate sunken guard cells. All BA Pb MEB 250.
9–10. Stomatal apparatuses inner surface. Arrows show guard cells partially preserved and arrowheads papillate subsidiary cells. All BA Pb MEB 251.

The five selected ratios among the three parts of the cuticle (the outermost part corresponding to the A1 layer, the middle part to the A2 layer, and the lower part to the B1 layer) were compared between *G. skottsbergii* and *G. ticoensis* using the Mann–Whitney test; among these 15 values (3×5), seven are significantly different and eight are homogeneous (Table 3). The most homogeneous ratio is N/Ca, which

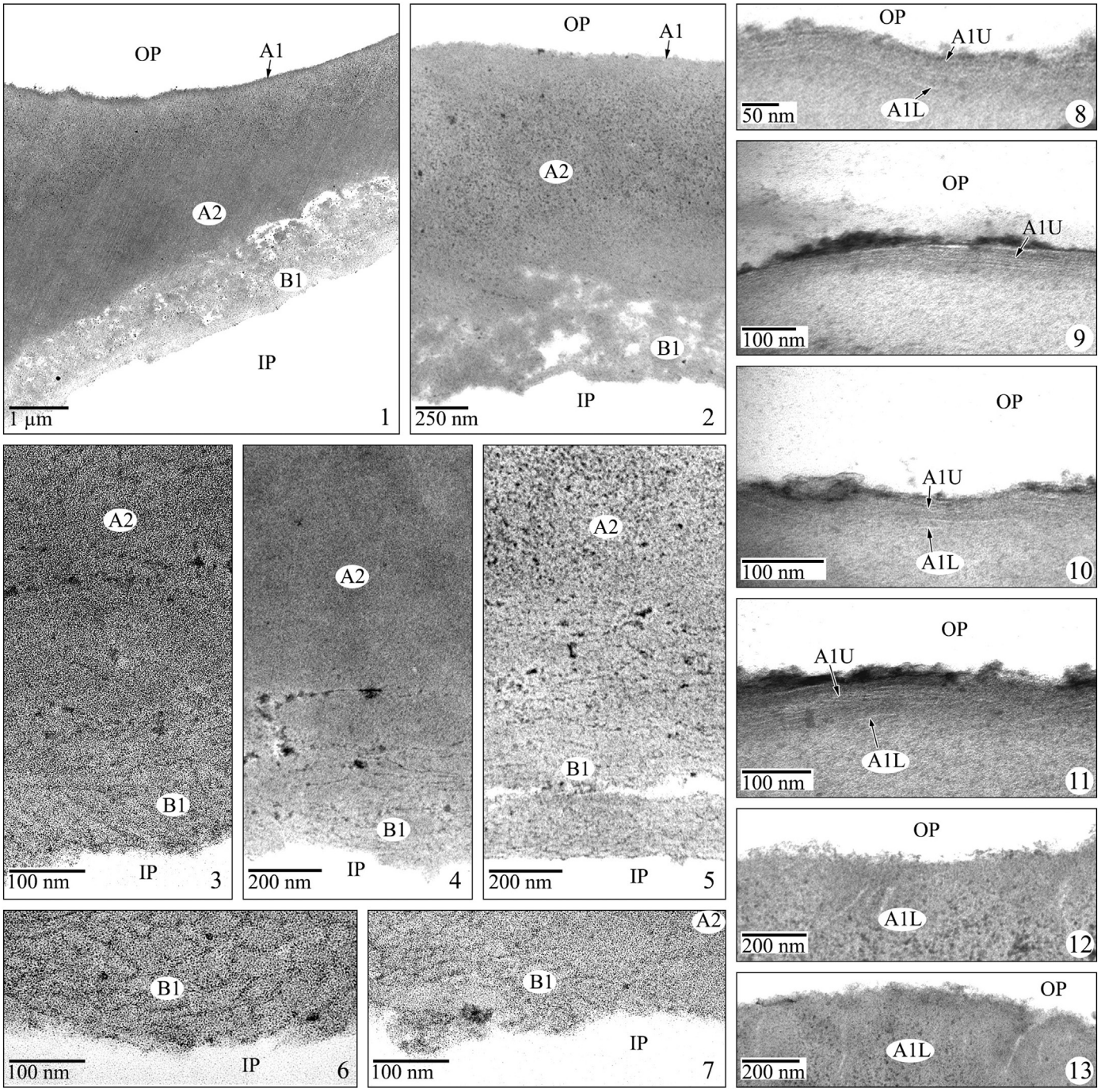


Plate IV. *Ginkgoites skottsbergii* Lundblad. Transmission electron microscopy of epidermal cell cuticle, general views and details. Transversal sections, except longitudinal section for photo 4. OP = outer part, IP = inner part.

1–2. General views of two parts, note the fibrilous B1 layer in the lower part of the cuticle, scarcely visible in these sections but with quite regular thickness.
3–5. Details of middle and lower parts, A2 granular layer being more or less contrasted.
6–7. Details of the lowermost part with reticulate fibrilous B1 layer in this case.
8–13. Details of the outermost part with A1 polylamellate layer, divided in two sublayers: straight A1 upper polylamellate part A1U with condensed lamellae arranged parallel to the outer surface of the cuticle, while the A1 lower polylamellate part A1L just underneath has more dispersed polylamellae even arranged perpendicular to the outer surface as in photo 12 and 13. In some rare cases, just one of the two sublayers is observed as in photos 9 and 12–13.

never exhibits differences between the layers of the two taxa and shows high standard deviations. The most heterogeneous is K/Ca, always exhibits differences. The other three are intermediate. Cl/N in A1 and A2 are homogeneous, while B1 exhibits differences between the two taxa. It is exactly the opposite for K/S, and S/Ca shows a heterogeneous A1 and homogeneous B1 and B2.

4. Discussion

4.1. Gross morphology, epidermal structure

Among ginkgoalean taxa *Ginkgoites* is perhaps the fossil genus whose species have been most subjected to repeated combinations.

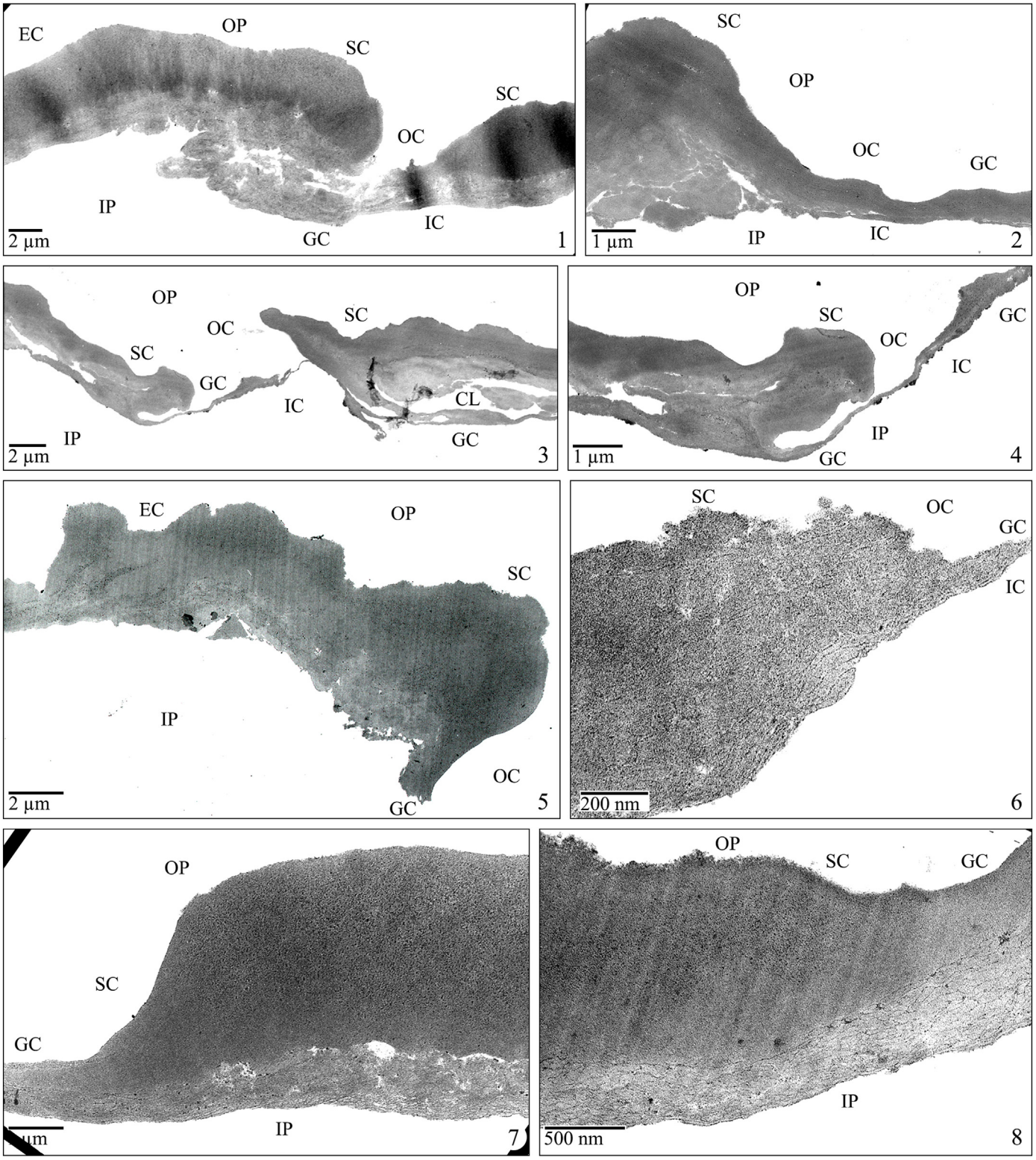


Plate V. *Ginkgoites skottsbergii* Lundblad. Transmission electron microscopy of stomatal apparatus cuticle, general views. Transversal sections. OP = outer part, IP = inner part; OC = outer chamber of the stomatal apparatus, in the upper part; IC = inner chamber, in the lower part; SC = subsidiary cell cuticle, GC = guard cell cuticle. 1–8. The shape and size of the different parts is different for each photo according to the location of the sections in each stomatal apparatus. 4 is a detail of 3.

their leaves were highly divided into narrow segments (Florin, 1936). On the other hand, Harris and Millington (1974) preferred the use of the generic name *Ginkgo* L. over *Ginkgoites* because they considered the latter genus to be ambiguously defined, whereas Czier (1998) regarded *Ginkgoites* and *Baiera* as synonyms of *Ginkgo* on the basis of posed by Seward (1919) and later emended by Watson et al. (1999), who retained the status of this taxon for fossil *Ginkgo*-like leaves for which the corresponding reproductive organs are unknown.

Leaf remains of species belonging to *Ginkgoites* or *Ginkgo* are very abundant in the Mesozoic worldwide; however, the structure and fine

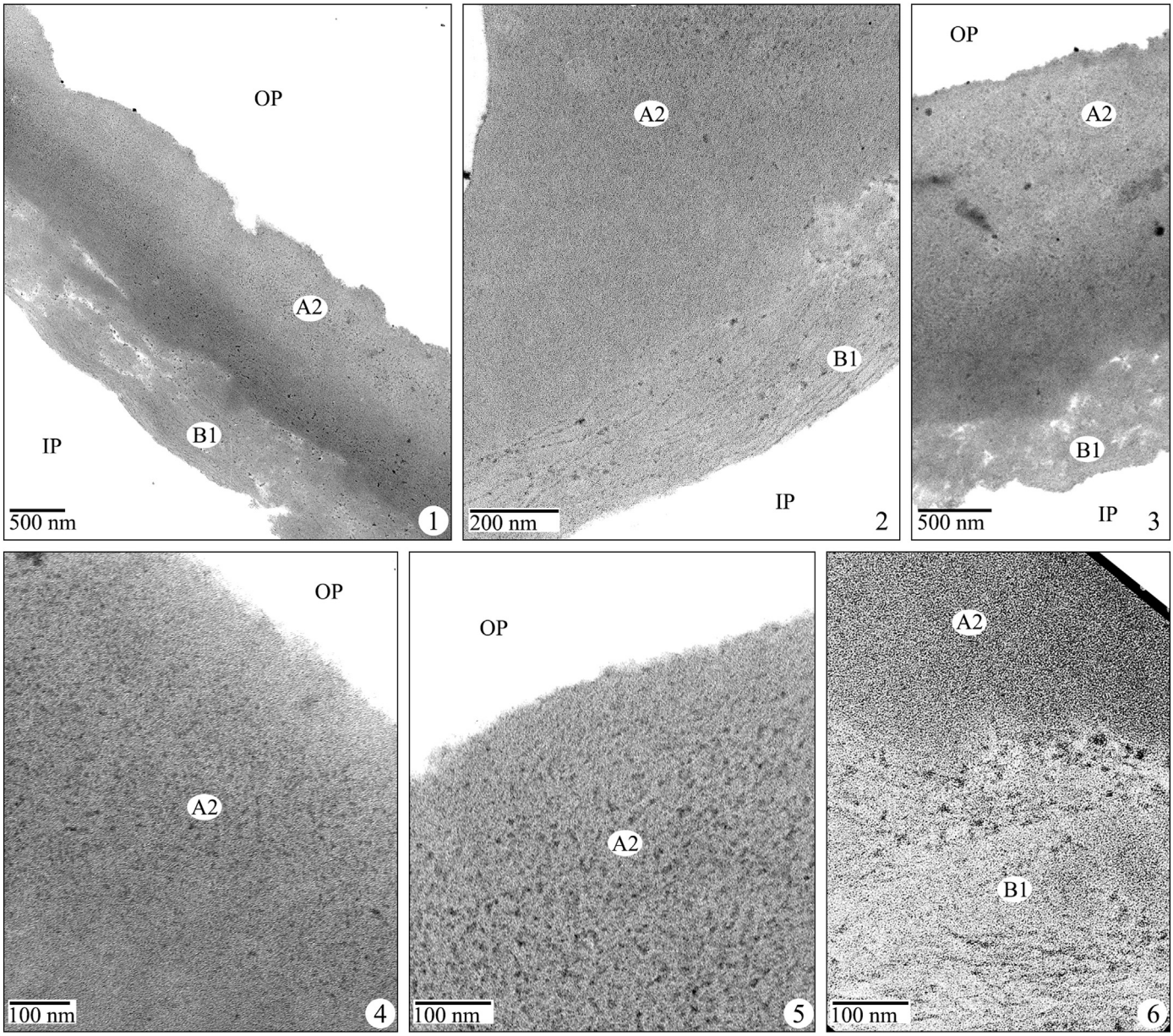


Plate VI. *Ginkgoites skottsbergii* Lundblad. Transmission electron microscopy of details of subsidiary cell cuticles. Transversal sections. OP = outer part, IP = inner part.

1–3. Lower magnifications showing the whole parts of the cuticle, the outermost part being devoid of A1 polylamellate layer. Granular A2 layer is very regular in consistency and B1 layer in the lower part is fibrilous or reticulate.

4–6. Higher magnifications showing various parts of the cuticle with heterogeneous B1 layer, fibrilous or reticulate.

structure of the foliar cuticle are well preserved in only a few taxa. In this study, new and relevant information has been obtained about the foliar cuticle of *G. skottsbergii* that reinforce the distinctions already established in earlier contributions among those *Ginkgoites* and *Ginkgo* species from the Cretaceous of the Southern Hemisphere. To avoid redundancy, the complete comparisons are available in Del Fueyo et al. (2013) and Villar de Seoane et al. (2015).

Therefore, the combination of both the morphological and epidermal foliar features observed in *G. skottsbergii* show that it is unequivocally different from any other *Ginkgoites* or *Ginkgo* species hitherto studied and highlight the specific identity of this taxon.

4.2. Palaeoenvironment at Bajo Comisión locality

G. skottsbergii was part of a plant community composed of bryophytes, pteridophytes, pteridosperms, bennettites, conifers and angiosperms according to megafloristic studies on the Kachaike Formation at the Bajo Comisión locality (Halle, 1913; Passalia, 2007a, 2007b). A recent depositional analysis of a complete sequence of this unit at the same locality suggested that the palaeoenvironment was dominated by deltaic sedimentation (prodelta–deltaic front–subaqueous plains), with a moderate marine influence in the lower part of the sequence and becoming typically continental (sub-aerial-fluvial) in the upper half of the section (Archangelsky et al., 2012). Furthermore, the plant assemblage recovered from this latter section was interpreted as open vegetation growing under conditions of high humidity on relatively nutrient-poor soils (Passalia, 2007b). A humid and warm climate was confirmed by Villar de Seoane and Archangelsky (2008), who found in the upper part of the Kachaike Formation at Bajo Comisión megaspore assemblages dominated by heterosporous lycopsids (Isoetaceae and Selaginellaceae), water ferns (Marsileaceae and Salviniaceae) and the existence of abundant water bodies. The discovery of tropical

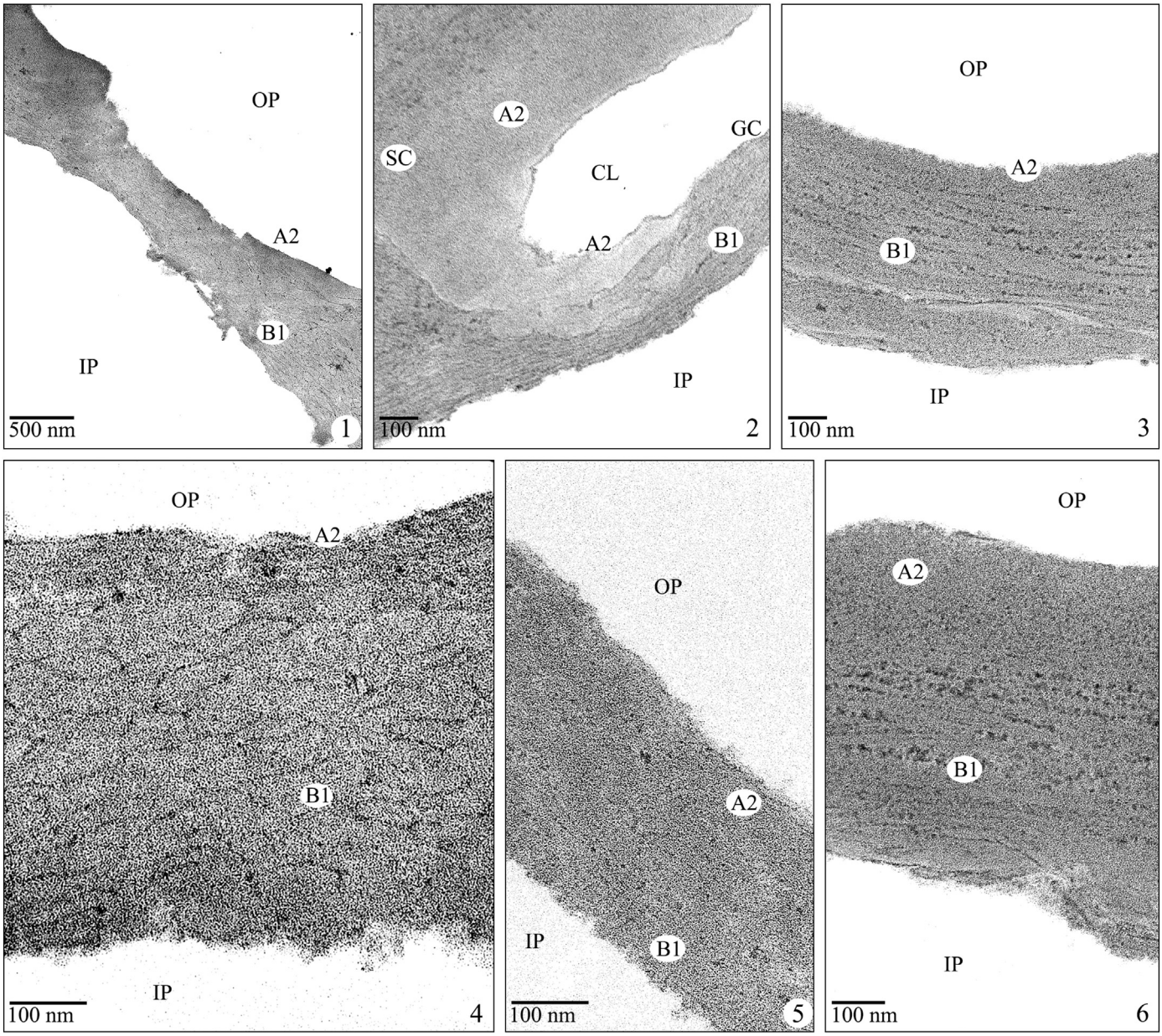


Plate VII. *Ginkgoites skottsbergii* Lundblad. Transmission electron microscopy of details of guard cell cuticles. Transversal sections. OP = outer part, IP = inner part. 1–6. The several sections show the absence of A1 polylamellate layer at the outer surface, A2 granular layer has various thicknesses and B1 fibrils in the lower part are very contrasted and thick in most cases, arranged parallel to the inner surface or making a reticulum.

angiosperm pollen related to basal groups of the Chloranthaceae, Winteraceae and aquatic to semi-aquatic herbs of the Alismatales revealed warm temperate conditions for that area (Barreda and Archangelsky, 2006).

The foliar features observed in *G. skottsbergii*, such as the low stomatal density and barely developed papillae that are restricted to subsidiary cells, may imply that this species was adapted to a mesic environment with a warm climate, adequate soil moisture and moderate to high humidity. Although the multilobate lamina, sunken guard cells and epicuticular waxes present in *G. skottsbergii* may be more related to arid environments, its whole set of morphological and cuticle foliar characteristics agree more closely with mesomorphic than with xeromorphic properties. If it is accepted that the parent plant of *G. skottsbergii* inhabited a region with an open vegetation and a warm climate, its compound leaves with smaller individual leaflets may provide greater ventilation and reduce overheating to maintain a favourable carbon and water balance, as was noted for such leaf-types by Wullschleger et al. (2014). Moreover, the amphistomatic nature observed in the leaf of *G. skottsbergii* may increase the maximum leaf conductance of CO_2 in response to a high light environment (Mott et al., 1982).

In contrast, other taxa from Patagonia such as *G. ticoensis* and *G. tigrensis* from the Aptian Anfiteatro de Ticó Formation show xeromorphic features (Del Fueyo et al., 2013; Villar de Seoane, 1997). The former taxon has papillae in both epidermal layers and papillate stomatal apparatuses with sunken guard cells, whereas the latter has epicuticular waxes and the subsidiary cells have a conspicuous ring around the slightly sunken guard cells. These two taxa grew in a floodplain with sinuous fluvial channels in a hot to temperate palaeoclimate. The xeromorphic features of these ginkgophytes are interpreted as adaptations against the high ash particle content in the atmosphere produced by the extensive and

Table 1
Ultrastructural values of *G. skottsbergii* based on 30 measurements.

	Ordinary epidermal cell cuticle				Subsidiary cell cuticle			
	Mean	Min–max	s-d	%	Mean	Min–max	s-d	%
CM	2.07	0.94–4.92	1.00	100	2.89	1.35–5.53	1.14	100
CP (A = A1 + A2)	1.46	0.68–3.64	0.69	70.5	1.88 (A2)	0.83–4.56	0.77	65.1
A1	0.05	0.025–0.078	0.016	2.37	Absence			
$A1_U$	0.025	0.005–0.046	0.011	1.21	Absence			
$A1_L$	0.024	0.010–0.057	0.010	1.16	Absence			
A2	1.41	0.65–3.60	0.69	68.1	1.88	0.83–4.56	0.77	65.1
CL (B = B1)	0.61	0.26–1.39	0.35	29.5	1.01	0.38–2.78	0.68	34.9
OL (nm)	13.08	4.17–30.31	5.81		Absence			
TL (nm)	4.83	1.75–9.34	1.86		Absence			
Nb TL	6.23	3–9	2.05		Absence			

	Guard cell cuticle			
	Mean	Min–max	s-d	%
CM	0.47	0.25–0.63	0.12	100
CP (A = A2)	0.15	0.07–0.27	0.06	31.9
CL (B = B1)	0.32	0.14–0.49	0.10	68.1

Note. % = percentage of the different layers and zones; min–max = minimum and maximum values of the mean, s-d = standard deviation. CM = cuticular membrane (CP + CL); CP = cuticle proper (A = A1 + A2); A1 = outer polylamellate layer of cuticle proper ($A1_U$ + $A1_L$); $A1_U$ = upper part of A1; $A1_L$ = lower part of A1; A2 = inner mainly granular layer; CL = cuticular layer (B); OL = opaque lamellae of the polylamellate layer (A1); TL = translucent lamellae of the polylamellate layer (A1). Except OL and TL (measured in nm), all other values are in μm.

recurrent volcanic activity that occurred during the sedimentation of the Anfiteatro de Ticó Formation (Archangelsky, 2001; Del Fueyo et al., 2013; Villar de Seoane, 1997). It must be noted that in the present study, considering the absolute values of S for the two taxa (Appendix D in the online version of this paper), *G. ticoensis* from a volcanic area has higher values for the A2 and B1 layers than *G. skottsbergii* from a non-volcanic area, although these values could be related to the palaeoenvironment.

The ultrastructural features of the *G. skottsbergii* foliar cuticle appear in accordance with a mesic palaeoenvironment, such as the A1 polylamellate layer having about half the percentage thickness (2.37%, Table 1) of the more xerothermic *G. ticoensis* (5.1%, Del Fueyo et al., 2013) and the thinner OL lamellae of the A1 layer in *G. skottsbergii* (13.08 nm) versus that in *G. ticoensis* (22.5 nm, Table 5). Although various interpretations exist about the outermost polylamellae of layer A1 (e.g., the *Agave americana* cuticle study of Wattendorff, 1992), Jeffree (2006) thought that these polylamellae "may constitute the main cuticular permeability barrier". Fischer and Bayer (1972) also considered them equivalent to the impermeable epicuticular wax in other living taxa (*Plantago major* and *Ardisia crenata*). Moreover, in the Cheirolepidiaceae, which typically occurred in xerothermic environments, a thick peripheral polylamellate zone (A1 layer) occurs very often in the cuticle proper [*Hirmeriella muensteri* from the Liassic of Franconia, Germany (Guignard et al., 1998), *Pseudofrenelopsis dalatzensis* (Yang et al., 2009), *Tarphyderma* and *Glenrosa*, both possibly belonging to the Cheirolepidiaceae (Archangelsky and Taylor, 1986; Srinivasan, 1992; Zhou et al., 2000), and *Suturovagina* (Mairot et al., 2014)]. Other ultrastructural mesic features are not very evident, such as the total CM thickness of *G. skottsbergii*, which is thicker than that of *G. ticoensis* (Table 5); a high thickness may be observed in some cases of a humid climate (Hill, 1998a, 1998b; Haworth and mcElwain, 2008a; Steinthorsdottir et al., 2011). Plants growing in peat bogs, climbers and halophytes – all growing in humid conditions – often have thick cuticles. In the case of *Ginkgoites*, a different equilibrium of the layers may be evoked for *G. skottsbergii* compared with the more xerothermic *G. ticoensis*: a thicker A2 (1.41 μm versus 0.37 μm for *G. ticoensis*) and a lower percentage of B (29.5% versus 58.8% for *G. ticoensis*). Although not the aim of this study, this seems to demonstrate various tendencies for the effect of volcanic gases on plants as in the case of living *Erica* and *Pinus*: "A tendency to a reduction in the fibrillar structure followed by an increase of the granular component has been noted between non-fumigated and fumigated leaves" (Bartiromo et al., 2012, 2013).

Three species described by Watson et al. (2001) from the Lower Cretaceous Wealden Flora of England are of particular interest for comparison. In *Ginkgoites weatherwaxiae* the stomatal apparatus is confined to the densely papillate lower surface, *Ginkgoites nannyoggiae* has a heavily papillate and ridged upper cuticle, and in *Ginkgoites garlickianus* the occurrence of papillae is restricted to the subsidiary cells of the stomatal apparatus. These ginkgoalean taxa were thought to be part of mixed communities with czekanowskialeans and needle-leaved conifers living in moister valleys on the upland Wealden massifs under a warm, seasonal Mediterranean Wealden climate with occasional equable humid periods (Watson et al., 2001).

Other examples of Ginkgoaceae are *Ginkgo shiguaiensis* Sun et al. and *Ginkgo longifolius* (Phillips) Harris reported from the Middle Jurassic of Inner Mongolia, China Sun et al. (2008), which have sparsely amphistomatic leaves, slightly sunken guard cells

Table 2
EDS comparisons between the layers within each of the two taxa of *Ginkgoites*.

		A1 layer	A2 layer	B1 layer
Ratio Cl/N	*G. ticoensis*	----------		
	G. skottsbergii	----------		----------
Ratio K/S	*G. ticoensis*	----------		
	G. skottsbergii	----------		----------
Ratio N/Ca	*G. ticoensis*	----------		

	G. skottsbergii	----------		
Ratio S/Ca	*G. ticoensis*	----------		

	G. skottsbergii	----------		
Ratio K/Ca	*G. ticoensis*	----------		

	G. skottsbergii	----------	----------	

Note. Within the two species, the significance of the distinctions/or not between ratios in A1–A2–B1 layers is illustrated with continuous or disrupted lines. It is checked with Mann–Whitney test with 5 measurements.

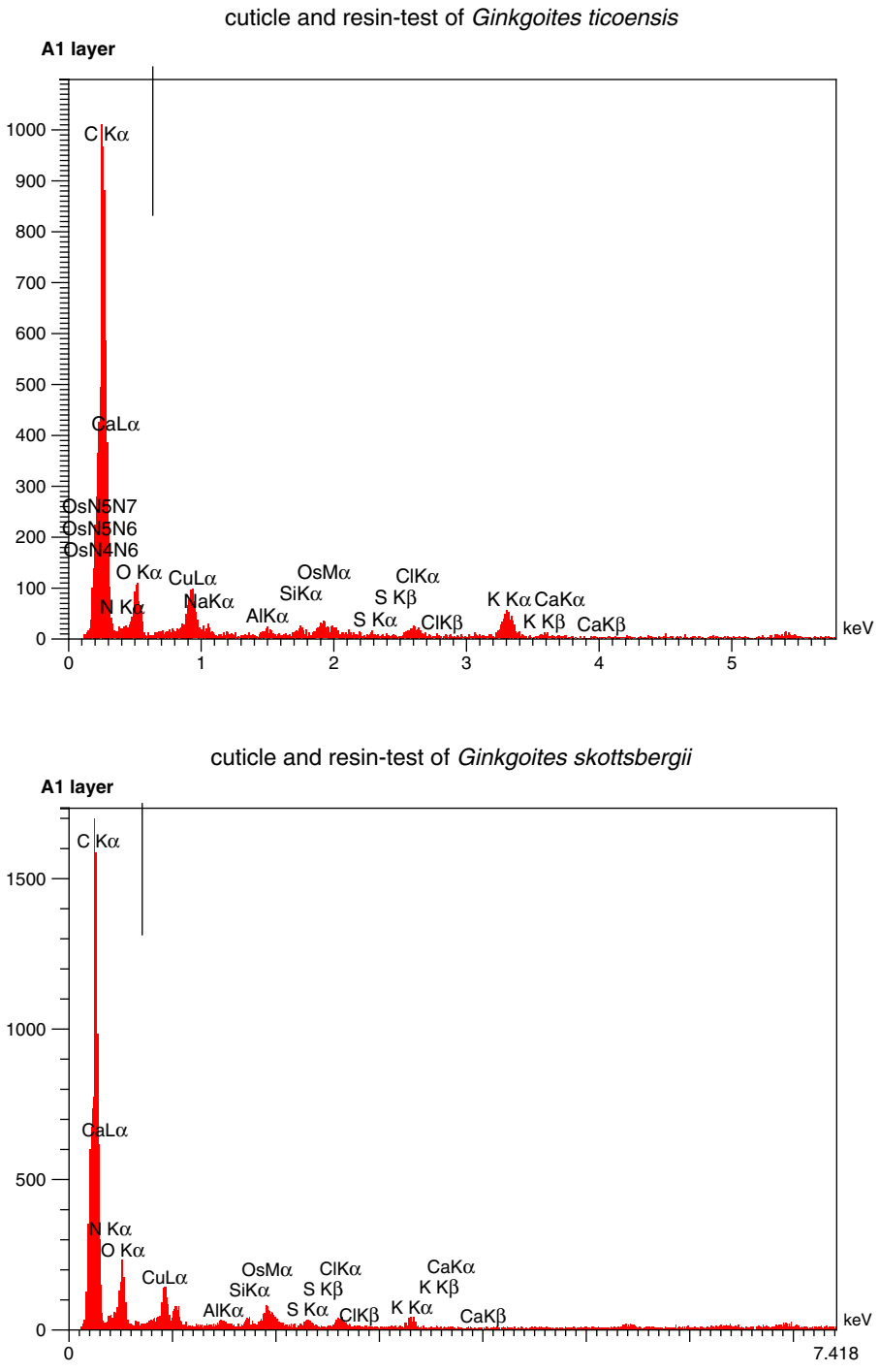


Fig. 2. Examples of EDS analysis.

and papillae only partially covering the stomatal pits. According to Sun et al. (2008), these plants grew along lake shores under a moist and warm to mid-temperate climate. *Ginkgo adiantoides* (Unger) Heer shows different foliar epidermal features depending on the localities and ages of strata from which they were recovered. Specimens from the Miocene of Greece and the Pliocene of Hungary have strongly papillate subsidiary cells, whereas those from the Pliocene of Germany are not papillate (Denk and Velitzelos, 2002). In the opinion of these authors, these marked differences between the fossil leaves from Greece and Germany may be due to ecotypical/intraspecific variability rather than to genetically fixed specific differences.

The fossil record has shown that during the Mesozoic, the Ginkgoales were mainly distributed in warm-temperate and mid-temperate regions, with seasonal variations and moisture conditions in both hemispheres. This is why Vakhrameev (1991) regarded them as a mesophilic group. In accordance with this, during the Cenozoic, fossil remains of *Ginkgo* were found mostly in moist and moderately warm conditions of the Northern Hemisphere (Uemura, 1997). Regarding the living *Ginkgo biloba*, which is widely cultivated around the world, it is also known that it has been conservative in its ecological tolerance because it is a light-demanding tree that grows well in exposed sites under warm and moist weather (Shan-An et al., 1997).

Table 3
EDS ratio comparisons between the two taxa of *Ginkgoites*.

			Mean	Minimum–maximum values	Standard deviation	Confidence interval	Significant differences or not, Mann–Whitney test
cuticle, A1 layer	*G. ticoensis*	ratio Cl/N	4.91	3.20–6.91	1.59	1.40	No, " genus " features
	G. skottsbergii		7.20	5.21–9.42	1.68	1.48	
cuticle, A2 layer	*G. ticoensis*		6.31	3.95–8.58	2.09	1.83	
	G. skottsbergii		4.97	3.95–5.84	0.85	0.75	
cuticle, B1 layer	*G. ticoensis*		6.65	4.28–9.28	2.18	1.91	Yes, " species " feature
	G. skottsbergii		12.86	9.54–17.59	3.72	3.26	
resin–test	*G. ticoensis*		53.41	11.40–125	51.38	45.01	No
	G. skottsbergii		24.34	11.11–54.80	17.86	15.66	
cuticle, A1 layer	*G. ticoensis*	ratio K/S	12.64	8.17–20.96	5.18	4.54	Yes, " species " features
	G. skottsbergii		1.94	1.59–2.38	0.34	0.30	
cuticle, A2 layer	*G. ticoensis*		9.83	7.32–12.98	2.51	2.20	
	G. skottsbergii		2.81	2.17–4.24	0.85	0.74	
cuticle, B1 layer	*G. ticoensis*		15.18	5.29–35.11	12.46	10.92	No, " genus " feature
	G. skottsbergii		8.47	5.79–11.71	2.30	2.02	
resin–test	*G. ticoensis*		2.68	2.20–3.20	0.40	0.35	No
	G. skottsbergii		2.57	1.77–3.86	0.83	0.73	
cuticle, A1 layer	*G. ticoensis*	ratio N/Ca	2.04	1.52–2.50	0.36	0.31	No, " genus " features
	G. skottsbergii		2.55	1.73–3.30	0.68	0.59	
cuticle, A2 layer	*G. ticoensis*		31.76	1.73–61	29.56	25.91	
	G. skottsbergii		9.18	3–32	12.76	11.18	
cuticle, B1 layer	*G. ticoensis*		50.95	5.75–70	25.67	22.50	
	G. skottsbergii		26.61	3.22–62	30.52	26.76	
resin–test	*G. ticoensis*		0.21	0.04–0.42	0.16	0.14	No
	G. skottsbergii		0.82	0.42–1.27	0.31	0.27	
cuticle, A1 layer	*G. ticoensis*	ratio S/Ca	2.95	1.83–4.50	1.17	1.02	Yes, " species " feature
	G. skottsbergii		6.53	5.36–7.90	1.08	0.95	
cuticle, A2 layer	*G. ticoensis*		55.09	2.93–108	51.90	45.49	No, " genus " feature
	G. skottsbergii		14.13	4.77–45	17.29	15.16	
cuticle, B1 layer	*G. ticoensis*		56.45	18–108	46.90	41.11	
	G. skottsbergii		17.45	1.78–51	22.32	19.56	
resin–test	*G. ticoensis*		0.85	0.70–1.00	0.12	0.10	No
	G. skottsbergii		0.73	0.58–0.81	0.11	0.09	
cuticle, A1 layer	*G. ticoensis*	ratio K/Ca	33.58	25.17–43.82	7.25	6.35	Yes, " species " features
	G. skottsbergii		12.39	11.73–13.50	0.84	0.74	
cuticle, A2 layer	*G. ticoensis*		473.23	33.95–808	402.31	352.64	
	G. skottsbergii		50.53	13.77–191	78.54	68.84	
cuticle, B1 layer	*G. ticoensis*		607.33	96.63–853	307.52	269.55	
	G. skottsbergii		172.42	13.89–597	253	221.76	
resin–test	*G. ticoensis*		2.24	2.00–2.53	0.23	0.20	No
	G. skottsbergii		1.80	1.44–2.25	0.31	0.27	

Note. Dark-orange colour means results for resin test, the two other colours being for the cuticle: light-purple for significant differences, light-brown for insignificant results.

Accordingly, *G. skottsbergii* may be an amphistomatic plant with a high photosynthetic capacity inhabiting a full-sun environment with continuously available soil water and having a high maximum leaf conductance. Therefore, this scenario may confirm that this species, as part of the open vegetation, lived under the humid and warm palaeoclimatic conditions suggested for the Bajo Comisión area during the Albian.

4.3. Elemental EDS analysis and ultrastructure

4.3.1. Elemental EDS considerations

As this is the first elemental data so far obtained in 2 species of the same genus belonging to the Ginkgoales, these results are unfortunately not comparable to previous data, but they allow discussion and could be a useful tool in future comparative studies. For the two species, *G. ticoensis* and *G. skottsbergii*, EDS analysis generated 15 new elemental features, with the evaluation of the five ratios among each of the three layers, A1, A2, and B1. Although the number of measurements is not great (five measurements for each value, due to the technique and time required for each measurement), most of these values are quite homogeneous and trustworthy (with a small standard deviation), the standard deviation being high for only a few values. Moreover, it is notable that within each cuticle of each taxon (Table 2), they show their own identity for the majority of cases (6), particularly for *G. skottsbergii*, while the three layers (A1, A2, B1) are homogeneous in only 4 out of 10 cases. Although elemental differences were not previously examined in fossil cuticles, this reflects the general variation in heterogeneity in the chemical composition of each layer already studied in living plants (Jeffree, 2006; Stark and Tian, 2006; Pollard et al., 2008; Dominguez et al., 2011). It also corresponds to the good quality of preservation of the fossil material that still show differences in the thickness of the cuticle after several tens of millions of years in the sediment. The influence of the sediment can be of course evoked; however, this would

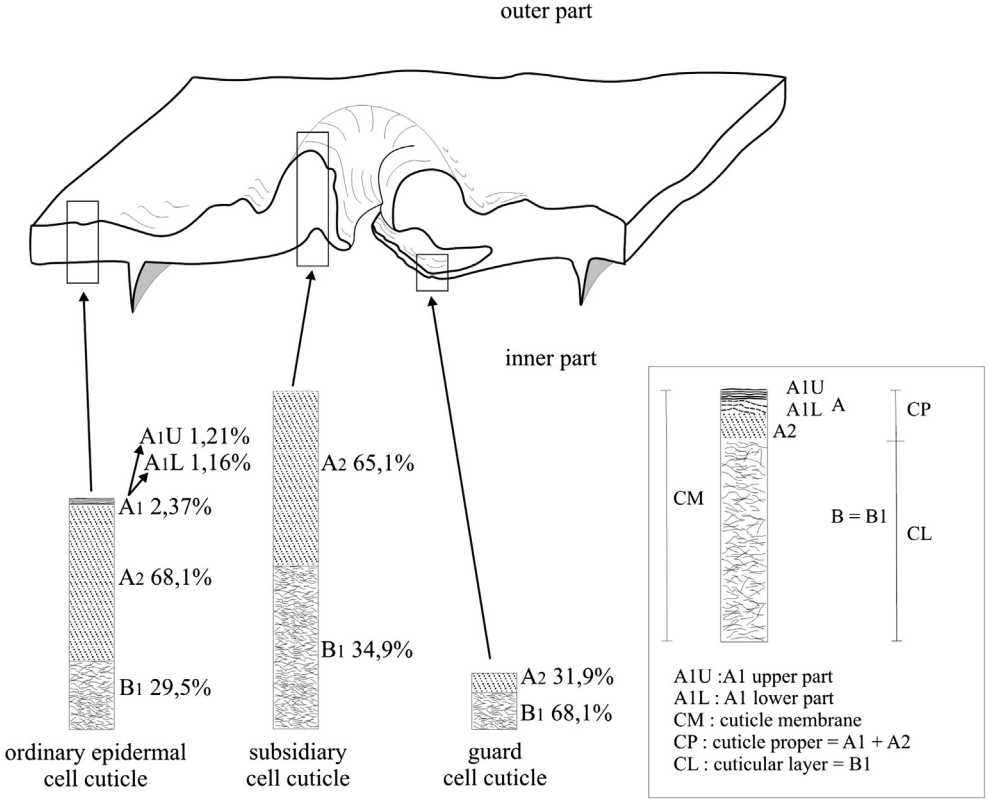


Fig. 3. 3D reconstruction of the cuticle.

Table 4
Key for the identification of each of the three types of cuticles of *Ginkgoites skottsbergii*.

A > B A = 1.5–1.9 μm, 65–70% A2 = 1.4–1.9 μm, 65–68%	→	Total CM = 2.1 μm A1 = 0.05 μm A1U = 0.025 μm A1L = 0.024 μm OL = 13.1 nm, TL = 4.8 nm B = B1 = 0.6 μm, 30%	→	Ordinary epidermal cell cuticle
		Total CM = 2.9 μm Absence of A1 layer B = B1 = 1 μm, 35%	→	Subsidiary cell cuticle
B > A A = A2 = 0.15 μm, 32%	→	Total CM = 0.5 μm Absence of A1 layer B = B1 = 0.3 μm, 68%	→	Guard cell cuticle

Note. The distinctions of measurements is using the confidence interval CI ($=\overline{x} \pm \sqrt{\frac{\text{var}}{n}} \times 1.96$. giving 95% α risk) with 30 measurements. In order to present easier criteria of distinction simplified values are provided in this table, they represent the mean or/and the percentage of the total CM for each layer or zone. The cuticular membrane CM is made up of cuticle proper CP (= A = A1U + A1L zones only present in ordinary epidermal cell cuticle, + A2 layer) and cuticular layer CL (= B = B1 layer). A1 layer (= A1U + A1L zones) is composed of alternate lamellae of opaque layer (OL) and translucent layer (TL). This table has to be read from the left to the right side.

probably correspond to a homogeneity of the elements throughout the cuticle, which is not the case.

The presence and the role of elements or more complex molecules in cuticles has been studied but is not yet fully understood (Dominguez et al., 2011; Fernandez and Eichert, 2009; Fernandez and Brown, 2013). Although some of these elements have been studied intensively, especially in the case of sulphur with respect to the environment and stomatal density for entire leaves (Haworth and McElwain, 2008a, 2008b; Haworth et al., 2012; Retallack, 2008), very few analyses of elements within the cuticles have been made. Bartiromo et al. (2012, 2013) studied in detail the cuticle layers with EDS from TEM sections; they showed that although sulphur is very common in volcanic fumaroles, it is totally absent from the cuticles of *Pinus* (a gymnosperm) and *Erica* (an angiosperm). However, in the case of fossil *Ginkgoites*, its presence in the two taxa could be related to two different palaeoenvironments or different metabolisms, as is generally inferred for living plants (Droux, 2004; Pautler et al., 2013). Calcium is present in two Orchidaceae taxa, although it is not related to their different cuticle thicknesses (De Oliveira Pires et al., 2003). It is also present in the ultrastructural details of the cuticles of the needles of *Picea* (Tenberge, 1992) and extant *Pinus* (a gymnosperm, Bartiromo et al., 2012), where it is related to the CaC_2O_4 of the volcanic activity in the vicinity of Mount Vesuvius near Naples, Italy. The nitrogen concentration has been studied in orchids (*Paphiopedilum* and *Cypripedium*) for adaptive significance together with the cuticle thickness (Guan et al., 2011) and it is also related to the cuticle thickness in *Laurus* and *Ceratonia* (Grammatikopoulos et al., 1998) and to the water permeability of the cuticle of ivy (*Hedera helix*, Santrucek et al., 2004). Potassium plays a role in plant cuticle development (genera *Agave* and *Clivia*, Wattendorff and Holloway, 1984), and its penetration through the cuticle has been studied in *Pyrus* (pear) and *Citrus* (Schönherr and Luber, 2001) and other angiosperms (Elshatshat et al., 2007; Schönherr, 2002), as is also the case for Cl (Schönherr, 2002).

In this first stage of our study, the palaeoenvironmental or other factors cannot be envisaged, but this elemental analysis provides some valuable taxonomic insights. Elements such as Al are used to distinguish between various parts of fossil remains (ovules, D'Angelo and Zodrow, 2011; hair-like structures, Zodrow, 2014; cuticles of tree-fern fossils, Zodrow et al., 2012). To compare the two species of *Ginkgoites*, the ratios of five elements were selected (N, K, Ca, S, and Cl). Although two species are too limited to provide a general view of the genus, the cuticles show

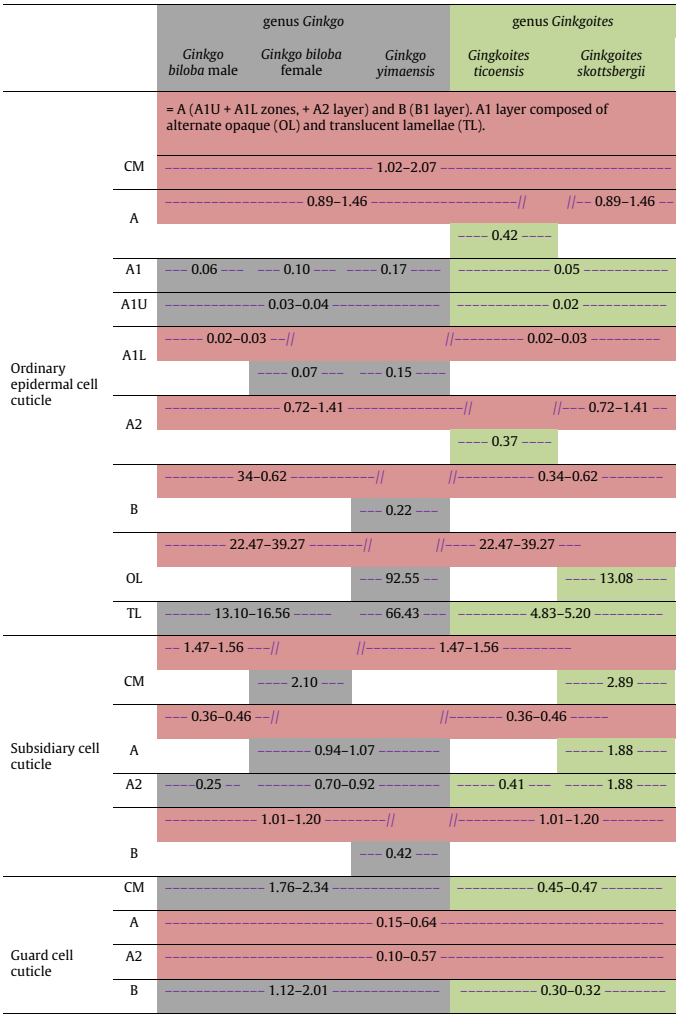
Table 5
First ultrastructural thicknesses and EDS ratio comparisons between two species of the genus *Ginkgoites*: *G. ticoensis* Archangelsky and *G. skottsbergii* Lundblad.

		Gingkoites ticoensis	*Ginkgoites skottsbergii*
Ordinary epidermal cell cuticle	Total CM	" species " feature: 1.02	" species " feature: 2.07
	Total A	" species " feature: 0.42	" species " feature: 1.46
	A1	" genus " feature: 0.050–0.052	
	A1U	" genus " feature: 0.023–0.025	
	A1L	" genus " feature: 0.024–029	
	A1 ratios Cl/N, N/Ca	" genus " features: 4.91–7.20, 2.04–2.55	
	A1 ratios K/S, S/Ca, K/Ca	" species " features: 12.64, 2.95, 33.58	" species " features: 1.94, 6.53, 12.39
	A2	" species " feature: 0.37	" species " feature: 1.41
	A2 ratios : Cl/N, N/Ca, S/Ca	" genus " features: 4.97–6.31, 9.18–31.76, 14.13–55.09	
	A2 ratios K/S, K/Ca	" species " features: 9.83, 473.23	" species " features:2.81, 50.53
	Ol (nm)	" species " feature: 22.5	" species " feature: 13.08
	Tl (nm)	" genus " feature: 4.83–5.2	
	nb of TL	" genus " feature : 5.80–6.23	
	B = B1	" genus " feature: 0.60–0.61	
	B1 ratios K/S, N/Ca, S/Ca	" genus " features: 8.47–15.18, 26.61–50.95, 17.45–56.45	
	B1 ratios Cl/N, K/Ca	" species " features: 6.65, 607.33	" species " features: 12.86, 172.42
Subsidiary cell cuticle	Total CM	" species " feature: 1.47	" species " feature: 2.89
	Total A	" species " feature: 0.46	" species " feature: 1.88
	A2	" species " feature: 0.41	" species " feature: 1.88
	B = B1	" genus " feature: 1.01	
Guard cell cuticle	Total CM	" genus " feature: 0.46–0.47	
	Total A	" genus " feature: 0.15–0.16	
	A2	" genus " feature: 0.14–0.15	
	B = B1	" genus " feature: 0.30–0.32	

Note. Comparisons between the two species of the genus *Ginkgoites* (*G. ticoensis* data from Del Fueyo et al., 2013) are evaluated by the confidence interval (ultrastructural thicknesses data with 30 measurements) and test of Mann–Whitney (EDS element ratios with 5 measurements). For the subsidiary and guard cell cuticles, as A1 layer is missing in *G. skottsbergii* these features are of course absent of this table. "Genus" features represent insignificant differences between the two taxa and the values are merged, while "species" features represent significant differences and they are separated. The values represent the mean, both for ultrastructural data and EDS elemental ratios. The cuticular membrane CM is made up of cuticle proper CP (= A = A1upper A1U + A1 lower A1L zones, + A2 layer) and cuticular layer CL (= B = B1 layer). A1 layer is composed of alternate opaque lamellae (OL) and translucent lamellae (TL). Except for very thin OL and TL measured in nm, all other measurements are in μm.

seven species features that difer significantly and eight genus features that do not (Table 3). Two comments can thus be made, one for the ratios and the other for the layers. First, among the five ratios, N/Ca exhibits only genus features in the three layers, while K/Ca is much more precise, exhibiting species features in all of the layers; the other three ratios (Cl/N, K/S, S/Ca) are intermediate. Second, all three layers show both species and genus features. However, there is a difference; the A1 layer is the most distinct between the two taxa with three species features (K/S, S/Ca, K/Ca), while the A2 and B1 layers each exhibit two species features (K/S and K/Ca and Cl/N and K/Ca, respectively). The eight genus features are mostly located in the A2 and B1 layers (Cl/N and K/S, respectively, with N/Ca and S/Ca being common to the two layers), while the A1 layer has two genus features (Cl/N and N/Ca).

Table 6
First ultrastructural comparisons between the genera *Ginkgo* and *Ginkgoites*.



Note. Ginkgos thicknesses data are from Guignard and Zhou (2005), *Ginkgoites ticoensis* data are from Del Fueyo et al. (2013). The data are evaluated by the confidence interval (with 30 measurements for *Ginkgoites* taxa, with 20 measurements for epidermal cell cuticles of ginkgos taxa, with 10 measurements for stomatal apparatuses cuticles of ginkgos taxa). The values represent the means, thin continuous lines indicate homogeneous values between the taxa, though with insignificant differences; disruptions in the lines indicate heterogeneous values with significant differences between the taxa. Light green colour indicates values significant just for *Ginkgoites* taxa, light blue colour indicates values significant just for *Ginkgo* taxa, light purple colour indicates insignificant and homogeneous values shared by the group *Ginkgo–Ginkgoites*. One qualitative feature is added for CM as the cuticular membrane CM is made up of cuticle proper CP (= A = A1upper A1U + A1 lower A1L zones, + A2 layer) and cuticular layer CL (= B = B1 layer). A1 layer is composed of alternate opaque lamellae (OL) and translucent lamellae (TL). Except for very thin OL and TL measured in nm, all other measurements are in μm.

Taxonomic features are considered in examining the fossilisation process and may include elemental studies (Zodrow and Mastalerz, 2009), but with much more complex biomolecules. The use of aromatic compounds and aliphatic residues for the study of paleosols in Canadian arctic material (Simpson et al., 2003, 2007; Kelleher and Simpson, 2006; Pautler et al., 2013) revealed a mainly angiosperm Quaternary fossil vegetation with a majority of dicotyledons and a minority of monocotyledons. More precisely, Al may be used as one of the elements to distinguish between genera and species (gymnosperms in D'Angelo et al., 2010; seed ferns, conifers and cycad-related fossils in D'Angelo et al., 2011; pteridophyll cuticles in Zodrow and Mastalerz, 2007; seed ferns in D'Angelo et al., 2012; Zodrow et al., 2009, 2010), as is also the case for N and Cl (tree ferns in Stoyko et al., 2013). Although still very limited to few taxa (2), the present elemental study in Ginkgoales has a potential interest for taxonomy, revealing both genus and species features, as well as variations between the different parts of the cuticle.

4.3.2. Ultrastructural and elemental EDS ratios of cuticle features at the levels of species, genus and the Ginkgo–Ginkgoites group within the Ginkgoaceae

The good quality of the material permitted statistical measurements and the use of confidence intervals with 30 values, a three-dimensional reconstruction of the cuticle (Fig. 3), and the identification of the three types of cuticles (the ordinary epidermal cell cuticle OEC, subsidiary cell SC and guard cell GC cuticles of the stomatal apparatus) for this species (Table 4). As this is the second statistical study using 30 statistical measurements within *Ginkgoites* (after the *G. ticoensis* data of Del Fueyo et al., 2013), comparisons can be made between the two fossil taxa (Table 5).

Including the EDS elemental results of the two taxa, among the 33 features provided, 14 are species features (seven EDS element ratios and seven ultrastructural features) that demonstrate the identity of each taxon and prompt two remarks. First, considering the three types of cuticle, the numbers of their features are not equivalent (25 for OEC including EDS elemental ratios, four for SC, and four for GC; these latter two cuticles have many fewer values, as they are devoid of an A1 layer and no EDS values are available). However, the subsidiary cell cuticle is revealed to be particularly important at the level of species recognition (3 out of 4 of its features), compared to the ordinary epidermal cell cuticle having only half the features of this type (11 out of 25) and none in the guard cell cuticle (0 out of 4). Second, considering the different cuticular layers, the total cuticular membrane CM and the total cuticle proper A and A2 layers are shown to be species features (except for GC).

Also at the generic level *Ginkgoites* is well-constrained as the species share 19 characters, eight being EDS elemental ratios and 11 ultrastructural features. Considering the three types of cells, the guard cell cuticle provides the best information about the genus *Ginkgoites* (4/4 of its features), followed by the ordinary epidermal cell cuticle with more than half of its features (14 out of 25); finally, the subsidiary cell cuticle has the least information about the genus (only 1 out of 4 of its features). Considering the different cuticular layers, the B layer represents the best information about the genus (3/3 among the three types of cuticles), followed by the A1 layer (in almost all its details: A1U and A1L sublayers, thickness and number of translucent lamellae TL), but only among the ordinary epidermal cell cuticles, as it is absent in the SC and GC of *G. skottsbergii*. Although the number of measurements was not as high as in *Ginkgoites*, comparisons (Table 6, Appendix F in the online version of this paper) can be made with ginkgos (living *G. biloba* L. males and females and fossil *Ginkgo yimaensis*; values from Guignard and Zhou, 2005). Among the data available for comparing the five taxa are 18 ultrastructural features (10 for OEC, four for SC, four for GC), of which six are representative of the genus level. OEC shows the best information on the genus (3 out of 10 of its features), followed by GC (2 out of 4) and then the SC (only 1 out of 4).

Within the Ginkgoaceae (order Ginkgoales), these ultrastructural comparisons show the identity of the *Ginkgo–Ginkgoites* group, all having a cuticular membrane, CM, made up of a cuticle proper, CP (= A = A1U + A1L zones, + A2 layer), and a cuticular layer, CL (= B = B1 layer). The A1 layer (= A1U + A1L zones) is composed of alternate opaque (OL) and translucent lamellae (TL). These characteristics seem to concern only the Ginkgoaceae because the only other known Ginkgoales cuticle ultrastructure is from *Sphenobaiera* (Karkeniaceae, one of the few families of the order Ginkgoales; Zhou, 1997, 2003, 2009; Taylor et al., 2009), which shows a very different cuticle with only the A2 and B1 layers (Wang et al., 2005). In the details of the three types of cuticle (Table 6), in addition to the small value of the guard cell cuticle that is usually observed in other studies in the

ultrastructure [Cheirolepidiaceae: *Pseudofrenelopsis dalazensis* in Yang et al. (2009), *Hirmeriella munsteri* in Guignard et al. (1998); Pteridospermales: genus *Dichopteris* in Thévenard et al. (2005), genus *Pachypteris* in Guignard et al. (2004)], the thickness of the subsidiary cell cuticle is more or less similar to the ordinary epidermal cell cuticle. The stomatal apparatus shows the identity of the *Ginkgo–Ginkgoites* group for two of its GC (2 out of 4) and OEC features (1 out of 10, surprisingly the total thickness of the CM), and none of its SC features (0/4). In addition to these four features for the *Ginkgo–Ginkgoites* group, eight features are overlapping with mixed affinities among the four species (2 *Ginkgo* and 2 *Ginkgoites*).

The ultrastructural characteristics observed in *G. skottsbergii* show differences from other taxa belonging to several other orders. In two genera of Czekanowskiales, *Arctobaiera* and *Phoenicopsis*, a cuticle proper A (A2) and a cuticular layer B differentiated into two sublayers are recognized (B1 and B2; Zhou and Guignard, 1998). In the Pteridospermales, only the granular layer A2 is observed in *Komlopteris* (Guignard et al., 2001) and *Dichopteris* (Thévenard et al., 2005), while *Pachypteris* (Guignard et al., 2004) has an upper cuticle composed of A (A1 upper and A1 lower; A2) and B (B1) and a lower cuticle with A (A1 and A2) and B (B1). *Ruflorinia* (Carrizo et al., 2014) is also very heterogeneous, with an adaxial cuticle composed of A2, B1 and B2, while the abaxial cuticle has just B1 and B2 from the cuticular layer. Three types occur in the Bennettitales: one in *Otozamites*, *Zamites*, and *Dictyozamites* (an outer lamellate layer and inner alveolate layer, 0.90–1.70 mm in width), one in *Ptilophyllum* and *Pterophyllum* (an outer alveolate or reticulate layer and an inner lamellate-reticulate layer, 2.45–3.25 mm in width), and one in *Cycadolepis* and *Williamsonia* (an outer reticulate layer and an inner lamellate layer, 2.35–5.75 mm in width; Villar de Seoane, 1999, 2001, 2003). Among the gymnosperms (sensu lato) where statistical TEM data are comparable with the present data, the fossil family Cheirolepidiaceae in the Coniferales has a cuticle proper A (very typical with wavy A1 and A2) and a cuticular layer B [with B1 and B2 in *Pseudofrenelopsis* (Yang et al., 2009), *Hirmeriella* (Guignard et al., 1998), and *Suturovagina* (Mairot et al., 2014)]. In living *Pinus* (family Pinaceae; Bartiromo et al., 2012), the cuticle is very different from the previous cheirolepidiacean taxa, as it is only composed of the B1 fibrillous layer from the cuticular layer.

As previously noted by Guignard and Zhou (2005) for *Ginkgo* and for *G. ticoensis* (Del Fueyo et al., 2013) and *G. skottsbergii* study, none of the cuticles are assignable to any single cuticle type in living plants described by Holloway (1982), and they show multiple affinities with his types 1, 2 and 3. However, for living *G. biloba*, Villar de Seoane (1997) found a resemblance to Holloway's type 3.

5. Conclusions

New and relevant information was obtained through an extensive cuticular analysis with SEM and TEM of the leaves of *G. skottsbergii*, which permits an unequivocal identification of this ginkgoalean taxon. The features identified in the epidermis of both the petiole and lamina, as well as those of the laminar cuticular membrane, are added to the original diagnosis of this species, reinforcing the concept of Archangelsky (1965) and Lundblad (1971) about its correct placement within the genus *Ginkgoites*. The TEM studies on the three types of cell cuticle also reveal the identity of this taxon based on the layer thicknesses and EDS ratios. Moreover, ultrastructural comparisons with other previously studied *Ginkgoites* and *Ginkgo* taxa could indicate species, genus and family features, at least for the *Ginkgo–Ginkgoites* group.

During the Early Cretaceous in Patagonia, two ginkgoalean lineages were already well established, the Karkeniaceae and the Ginkgoaceae, represented by *G. tigrensis* Archangelsky and *G. ticoensis* Archangelsky, respectively. In this contribution, a new Patagonian member, *G. skottsbergii*, is added to the Ginkgoaceae, highlighting Patagonia as a region with a remarkable diversity. In addition, these three *Ginkgoites*-type leaves have been completely characterized because of their well-preserved epidermal features that clearly show the distinctions among them.

The sedimentology of the plant-bearing strata along with the associated flora composed of heterosporous lycopsids, water ferns and tropical angiosperms related to basal groups of the Chloranthaceae, Winteraceae and aquatic to semi-aquatic herbs of the Alismatales suggest the parent plants of *G. skottsbergii* as part of an open vegetation living during the Albian under the warm and moist palaeoclimatic conditions of the Bajo Comisión locality. Moreover, *G. skottsbergii* is regarded as a mesophytic plant with a high photosynthetic capacity and a high maximum leaf conductance inhabiting a full-sun environment with continuously available soil water.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.revpalbo.2016.05.002.

Acknowledgements

This study was supported in part by grants CONICET PIP 212/2012 and PICT 528/2012. The authors are grateful to Hans Kerp Editor-in-Chief of Review of Palaeobotany and Palynology and to the two anonymous reviewers for their comments and constructive suggestions that have greatly improved the manuscript. We thank Orlando Cárdenas for technical assistance in sample processing and Amalia González for drafting Fig. 1. We wish also to thank the technical staff of the University Lyon 1 "centre technologique des microstructures CTμ", particularly Xavier Jaurand and Béatrice Burdin for EDS analysis and statistics, and Sophie Cros for preparing all the technical work on the TEM plates and figures and supplementary data.

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