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Thirty-three years (1986–2019) of fossil plant cuticle studies using transmission electron
microscopy: a review

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Abstract

Although the cuticle is a very thin layer of a few micrometers, it is one of the main remains
found among fossil plants. Apparently very fragile, ultrathin cuticle sections of few dozen
nanometers thick are studied by using TEM studies and statistical measurements. The
ultrastructure details yielded are of significance not only for taxonomy, but also for
palaeoenvironment and evolution of fossil plant. After an historical introduction and a review
of the methods to obtain and observe the ultrathin sections, although data are still not enough

numerous, detailed information and interesting facts of cuticle ultrastructure emerge from the studied plant groups. This paper gives a review of the main results of transmission electron microscopy -TEM- of fossil plant cuticles since the pioneering study 33 years ago.

Keywords: fossil plant cuticles; ultrastructure; transmission electron microscopy TEM; taxonomy; palaeoenvironment; evolution

1 Introduction

1.1 Historical insights

Our knowledge of the plant cuticle, including its naming, spelling, and wording (coticola, cuticula, kutikula, cuticule, cuticle, etc.), progressed very slowly throughout centuries and countries. It began with the term ‘cuticle’ used in a very wide sense for plants and/or humans and animals, including formerly for plant cellular layer(s) beneath (epidermis and others...), towards the sensu stricto ‘plant cuticle’ used in a modern sense as an extracellular membrane over (or mixed with) the epidermis cell wall. The primary research to date on the subject, besides probable omissions, has been discussed here.

The term cuticle originates from Latin (*cuticula* = *cutis* + *cula* = thin skin or small skin). Identification of the outer parts of plants, or more precisely, allusions to the cuticle exist from ancient Greek and Latin periods; however, it was not named ‘*cuticula*’. The French term ‘cuticule’ was cited by Amigues’s Greek-French translation (1989) for the outermost part of the oak acorn base part (pro parte for the Greek verb παραλιθάζειν, which means ‘make stony around’) Luccioni (pers. com.), in the research on Theophrastus *Historia plantarum* (Book 3-8-3, p. 22; see also Amigues 1999). The English term ‘cuticle’ was not found in the Greek-English translation of Hort (1916), where ‘become stony at one end or the other’ was used. However, in this English translation, Theophrastus’ Greek term derma (δέρμα, = skin) has been translated to ‘skin’ when referring to the outer part of the olive (p. 399, Theophrastus Book IV XIV 9–10), whereas it has been translated to ‘outer covering’ for the outer parts of fruits in the general presentation of plants (p. 21, Theophrastus Book I 2 6; Luccioni pers. com.). The particular impermeable property of the cuticle (without naming it) is clearly noted in Book VII 141, for the frond of a fern (οἶον τὸ ἐπὶ τοῦ ἀδιάντου συμβαῖνον·νοῦδὲ γὰρ

64 ὑγραίνεται τὸ φύλλον βρεχόμενον, ὃ δὴ ἐπίδηλόν ἐστι διὰ τὸ μὴ τὴν νοτίαν ἐπιμένειν, ὅθεν
65 καὶ ἡ προσηγορία. γένη δὲ αὐτοῦ δύο, τὸ μὲν λευκὸν τὸ δὲ μέλαν, χρήσιμα δ' ἀμφοτέρω πρὸς
66 ἔκρυσιν κεφαλῆς τριχῶν ἐν ἐλαίῳ τριβόμενα). This text is translated into English as ‘...for
67 instance that which we find in wet-proof (maidenhair); the leaf does not even get wet when it
68 is watered, nor does it catch the dew, because the dew does not rest on it...’ (Hort, 1916, Vol 2
69 p. 135), whereas the French translation of Amigues (1989) is slightly different (Luccioni pers.
70 com): ‘...l’humidité n’adhère pas à sa surface...’ (= humidity does not adhere to its surface);
71 however, both translations imply the outermost part of the frond has impermeable properties.
72 Amigues (1996) commented on this fern, also used in Theocritus’ Idylls XIII poem (see also
73 Banks and Chapman 1853), and explained that the Greek name ἀδίαντον means ‘not wet’ and
74 uses the French words ‘cuticule cireuse’ (= waxy cuticle) in the comments. Although this is
75 not directly related to our subject, this plant seems somewhat difficult to identify as several
76 fern taxa have been proposed by the different translators; however, the species is very
77 probably *Adiantum capillus-veneris* L. (Luccioni pers. com.). This taxon is among the rare
78 ferns where the cuticle has been analysed using freeze-fracture (Wada and Staehelin 1981).
79 The term ‘cutis’ (= skin) has been used in ancient Latin texts: e.g. Pliny the Elder’s *Historia*
80 *naturalis* [Latin-French translation by Littré (1877)] used this term for the outermost part of
81 the oak acorns (Book XVI 8 2): ‘*Distant inter se magnitudine, et cutis tenuitate*’ (they differ
82 between them according to their size and the fineness of their skin). Columella [Latin-French
83 translation of *De re rustica* by Du Bois (1844), Lib. III], from that period too, also used this
84 term for the outermost part of a variety of grapes: ‘...*nam quia minuti acini, et durae cutis*
85 *uvas habent...*’ (because they have grapes with small grains and a thick skin). The oldest
86 Latin lexicons (Furno 1997) from the Roman period or a little later [Varron, from 1 BC (see
87 the edition of Goetz and Schoell 1910); Festus and Festo, from the IInd and VIIIth century
88 taking in account the text of Flaccus ‘*De uerborum significatu*’ from 1 BC (see Lindsay

89 edition of 1913); Marcellus, from the IVth century (see the edition of Lindsay 1903); Isidore
90 of Seville, from the VIth century (see the edition of Lindsay 1911)], do not cite ‘*cuticula*’. For
91 instance, the text of Isidore of Seville contains a whole section on plants, but only mentions
92 ‘*cutis*’ (Liber XVII–VIII 7 and IX 104, *De rebus rusticis – De aromaticis arboribus* and *De*
93 *herbis aromaticiis*).

94
95 From the XIth century, with a great increase from the end of the XVth to the XVIth century,
96 ‘*cuticula*’ appeared increasingly in the first general dictionaries of various languages in
97 western Europe (or glossaries, lexicons, or equivalents), but is mostly linked with ‘*cutis*’ and
98 human and/or animal skin, whereas it was used more accurately for botanical purposes in
99 more specialised texts. In the ‘*Elementarium*’ of Papias from the XIth century (see the edition
100 of Mombricitus 1485 or older manual versions), ‘*cuticula*’ was noted as a diminutive of ‘*cutis*’,
101 with a human and animal skin meaning. In 1286 (see an edition of 1460, by Gutenberg?),
102 Balbus used the same meaning. In one of the most widespread dictionaries of that period,
103 ‘*cuticula*’ was cited by Calepino (1502) as a diminutive of ‘*cutis pellis*’ [among numerous
104 uses of this text through almost three centuries, for instance in 1588 an English translation of
105 ‘*cuticula*’ was provided in eight languages (‘a little or thinnne skinne’; p. 176); in the ninth and
106 last edition of this text (Facciolatti 1778), ‘*cuticula*’ was still cited (p. 197) with a human
107 meaning]. In the *Thesaurus linguae latinae* of Estienne (1532, also edited in 1573), ‘*cuticula*’
108 was also noted (p. 707) for a diminutive of ‘*cutis*’, *cutis* referring in one case to plants (‘*cutis*
109 *arboris*’). The Latin word ‘*cutis*’ was cited once in the index of the Greek-Latin translation of
110 Theophrastus *Historia plantarum* by Theodorus Gaza (1552 edition, from the end of the XVth
111 to the beginning of the XVIth century). Scaliger (1644, p. 878) in the Greek-Latin commentary
112 on Theophrastus *Historia plantarum*, in the text corresponding to the fern cited above for its
113 impermeability, cited Theodorus Gaza's translation (similar sentence in Gaza 1552 p. 153):

114 “*Adianti, id est, capilli Veneris, folium numquam madescit, nec quicquam adhaesisse humoris*
115 *constat* (= *Adiantus*, i.e. *Capillus veneris*, never gets wet, and it is well-known that no liquid
116 is adhering to it”; Luccioni, pers. com). In the early XVIIth century, the diminutive *cuticula*
117 appeared in other western Europe dictionaries as a synonym of ‘coticola’ in the Italian
118 ‘vocabolario degli accademici della crusca’ [(1612), p. 150 of the ‘indice delle voci e
119 locuzione latine’ part (index of the Latin voices and locutions)]. ‘*Cuticula*’ was used several
120 times by Scaliger (1644), but as a wide definition of the outer part of plants, particularly for
121 the outer part of the fruit of *Paliurus spina-christi* (= garland thorn, Rhamnaceae; p. 266,
122 comm. on Theophrastus Book III 17; Luccioni pers. com). ‘Cuticle’ in English appeared in
123 one of the first English dictionaries (1658, ‘The New World of English Words, or, a general
124 dictionary’) but as an anatomical term for the human (or animal?) skin outerpart, and
125 appeared more than 10 times in Grew (1672), apparently referring to different external parts
126 of plants. ‘*Cuticula*’ was cited about 20 times in Malpighi (1675; also, *cuticulam*, *cuticulae*,
127 *cuticulâ*) apparently connected with different outer parts of plants, but specifically with
128 ‘*epidermide*’ (epidermis) and “*folii*” (leaf) in some parts of the book. In other western
129 European languages, the French dictionary of Furetière (1690) cited ‘cuticule’, but as a
130 synonym of the epidermis of animals. In the Portuguese dictionary of Bluteau (1712; p. 648),
131 although it was partly devoted to botany, ‘cuticula’ was a synonym of ‘cutícula’ and described
132 anatomically as human epidermis. Similarly, in 1729 ‘cuticula’ and ‘cuticular’ appeared in the
133 in the Spanish ‘Diccionario de Autoridades’ with the same meaning.

134
135 The plant cuticle has been described increasingly precisely and in detail through time; in
136 Ludwig (botanist and doctor, 1742), the plant cuticle was cited several times and described in
137 Part II *Consideratio vegetabilium physica* (Chapter II paragraph 315: ‘*est lamina densa,*
138 *tenuis, pellucida... et totam plantae superficiem obtegit... haec cuticula plantarum dicitur*; it

139 is a dense, thin, transparent layer... and it covers the whole plant surface... it is called plant
140 cuticle'; partly Luccioni (pers. com). It was compared with animal cuticles (paragraph 316),
141 and also quoted in the index. In other paragraphs (431 and 432), maceration experiments on
142 various taxa were described, separating the cuticle from parenchyma tissue. Some years later,
143 Ludwig (1757), described the cuticle slightly differently and also included maceration, which
144 possibly represents one of the first experiments on plant cuticles, but the term was still
145 apparently used in a wide sense ("*Omnem plantae superficiem cingit lamina tenuis et*
146 *pellucida, quae cuticula dicitur, haec densa est et in maceratione plantarum non*
147 *dissoluitur...*"; paragraph 327; "the whole plant surface is surrounded with a thin and
148 transparent layer, called cuticle, it is dense and not soluble by plant maceration..."; partly
149 Luccioni pers. com), further in this paragraph the cuticle was precisely restricted to some
150 parts of plants. It appears several times in this 1757 text, also compared with human skin in
151 the paragraph 328 ("*cuticula plantarum 327. Huius et humanae comparatio 328*" as written in
152 the index).

153
154 Linked to more precise techniques and experiments, the early XIXth century was the starting
155 point for various steps recognizing the plant cuticle as it is currently defined. Treviranus
156 (1821) provided transversal sections ('durshnitt') of various extant plants, but unfortunately
157 with low magnifications where the cuticle was difficult to distinguish from the cell wall of the
158 epidermis (his Tab. I F. 1, 4, 13; his Tab. II F. 14 and 21). However, this was an important step
159 for observing details of the different parts of the outer part of the epidermis, and the term
160 'cuticula' was used, but not in its modern sense. In 1827, De Candolle used the French term
161 'cuticule' in Chapter V of his book ('de la cuticule et de l'épiderme' p. 66–77; = about cuticle
162 and epidermis), where he referred to transversal and longitudinal sections of *Tritomia varia*
163 (his Planche 2 Figs. 3 and 4). In Figure 3, he clearly called cuticule (named 'c' on the figure)

the epidermal cells surrounded with a thick cell wall, the cuticle thickening not visible. In the text, he included epidermal cells in the ‘cuticule’, but cited other authors suggesting that the cuticle seems to depend on the type of organ and taxon observed. Brongniart (1830) used the same technique and provided, at a much higher magnification, very precise light microscope figures of extant plant leaves with transversal sections (also called perpendicular sections: ‘coupes transversales’, ‘coupes perpendiculaires à la surface... des feuilles’) obtained with very sharp and delicate instruments (‘...avec des instrumens très-tranchants et très délicats...’), where the cuticle seems to be drawn; however it is not clear for some cases if he drew the cuticle or cell wall. Although the term cuticle was not used, it was drawn quite precisely in the plates with extant *Lilum album* (Pl. 8 Fig. 3) and *Iris germanica* (Pl. 9 Fig. 2), showing a thicker thickening over the upper epidermis, whereas it was thinner in the lower epidermis. As other palaeobotanists of that period, Brongniart was very sensible to the quality of the illustrations (Cleal et al. 2005), which were accurately observed and illustrated, as he also researched extant immersed plants, such as extant *Potamogeton perfoliatum* and *Ranunculus aquatilis* that have almost no thickenings above the epidermal cells, which is well-known for plants in these environments. Brongniart referred to a continuous membrane (‘membrane continue’), except in the middle part of the stomata, which was obtained with extant *Brassica* leaves, after maceration in water for a few months.

Henslow (1831) published some clear cuticle preparations (without using this term) in extant *Digitalis* (Scrofulariaceae) hybrid organs (his Plate XVII 6–7; transversal sections in Plates XVII–XVIII also show cuticles over the epidermis) using nitric acid (an efficient technique still currently used for preparations of cuticles), and wrote: “I am however quite positive upon another point which has been a subject of dispute among physiologists; I mean the existence of a delicate homogeneous membrane investing this epidermis. Such a membrane may be

189 distinctly separated by the action of nitric acid, from the epidermis of the corolla, filament,
190 and style. It is faintly marked by parallel longitudinal stria... and appears to coat over the
191 whole surface of these organs...” Brongniart (1834) cited Henslow in his text related to the
192 *Brassica* experiment, but more accurately described the possibility of chemical maceration to
193 detach a thin ‘pellicule sus-épidermique’ (= film over the epidermis) from the epidermis (=“cuticle” in his text); according to his text he observed this in many other taxa. Lindley
194 (1835), published a whole part (Chapter II Section I 1; see also Plate III Figs. 3–6 and 9–11)
195 in the second edition of a botany book dedicated to ‘cuticle’, and summarised the research he
196 questioned about the cuticle as follows: “There is some reason to suppose that there is
197 occasionally present, on the outside of the cuticle, a transparent, very delicate membrane,
198 having no organic structure, as far as can be discovered with the most powerful microscopes”.
199 Something similar was noticed by Brongniart in the cabbage leaf; an analogous structure was
200 commented on by Henslow in the *Digitalis* leaves; “and I have found it very conspicuous on
201 the upper side of the leaves of *Dionaea muscipula*... Brongniart has paid some attention to
202 this subject... and finds the pellicle by no means uncommon; he even thinks it is present in
203 submersed leaves, and imagines that it covers the stigma in some plants. I, however, find
204 nothing very definite in regard to this, except that the pellicle often exists, and that it does not
205 cover the stomates”. The different editions of this book are of interest as they represent an
206 accurate reflection of the research regarding cuticles of that time. The first edition also
207 contained a ‘cuticle’ part (Lindley 1832), which was not very detailed. In the third edition
208 (Lindley 1839) the ‘cuticle’ part was removed and replaced by an ‘epidermis’ part, where the
209 cuticle was described to be ‘organic mucus’. His Plates (I Fig. 2 and II Fig. 1) contained some
210 transversal sections but with the cuticle very simply drawn on the outer part of the epidermis.
211
212
213 Von Mohl (1842, 1847a) made a considerably more precise new finding, showing the

different parts of the cuticle (especially the presence of a ‘cuticle proper’, as cited by Holloway 1982) and simultaneously used the German term ‘cuticula’ (= kutikula) in its modern sense. The translation of the 1842 article in French (Von Mohl 1843) seems to be the first appearance of the French term ‘cuticule’ in its modern sense, and contained a detailed illustration (Plate 6) with various coloured sections of cuticles, two of them being clearly bilayered (his Fig. 7 of *Viscum album*, his Fig. 10 of *Agave lurida*), other figures being from mono- to multi-layered; interestingly for Coniferales, which are now among the well studied fossil plant groups using transmission electron microscopy (TEM), the cuticle of extant gymnosperm *Taxus baccata* was mentioned (as similar to his Figure 8). Hartig (1843) published extant cuticles schemes of sections with 1 and 2–3 zones (his Pl. Figs. 1 and 2), and a whole chapter entitled ‘cuticule’ in the translation in French of this article (Hartig 1844). In the French translation (Von Mohl 1847b) of the article of Von Mohl (1847a), there was a description of the use of transversal sections to determine the chemical reactions of the cuticle. However, these new cuticle findings (several parts for the cuticle) seemed to have a variable impact according to the authors. From Lindley’s botany book, the fourth edition (1848) contained ‘back’ the ‘old wide sense’ of cuticle in much more detail than the first and second editions, including the current research of this time, and the cuticle was compared with the ‘skin or shell of Crustaceae’. With Henfrey’s English translation (1852) of von Mohl (1851) the English term cuticle in its modern sense (‘... mass secreted on the outside of the cells, the true *cuticle*... in which no cellular has been yet shewn to exist’) appeared seven times. Transversal sections were provided (Figs. 17, 37, 40–42, 48) where the cuticle was indicated in the legends by ‘a’ (Figs. 37, 41, and 48 of extant *Helleborus*, *Hoya*, and *Cereus*). Moreover, his Fig. 41 was particularly informative, as the cuticle was very clearly shown as detached from underlying layers, and corresponded to the cuticle proper in its modern sense, the cuticular layer in its modern sense just underneath being named ‘b’ (‘wollen, laminated

239 layers of the epidermis cells’); the cell wall was present all over the cell and very precisely
240 drawn.
241
242 Bornemann (1856) published some transversal sections (‘Querschnitt’) of various extant
243 cycads (his Tafel XI 18–19; his Tafel XII 15–18 with two parts in Section 17 above the
244 epidermis cells, one for the cell wall, and one for the cuticle with a clear anticlinal wall
245 between the cells). In the same year Schacht (1856) provided coloured transversal sections
246 (his Tafel III) of extant plants with cuticles showing various thicknesses, distinct from the cell
247 wall just beneath in another colour; in one drawing the cuticle had two parts, an outermost
248 dark-brown and an inner yellow part. In addition, also in the same year Prillieux (1856)
249 published (his Pl. 2 and 3) some sections of various Oleaceae and Jasmineae, where the
250 distinction between the cell wall of the epidermis and cuticle was not very clear. Unger (1858)
251 provided a transversal section of extant *Richardia aethiopica* (his Taf. I 4) with a cuticle
252 apparently drawn in a darker colour than the cell wall, but in a very low magnification, not
253 showing any details of the cuticle. Besides, much earlier, this author had published the same
254 results for extant *Victoria regia* leaf (Unger 1853, his Figs. 2–3). Wolf (1865) provided
255 transversal sections of the epidermis of *Orchis mascula* and *Trichopilia suavis* (his Tafel XVI
256 3 with ‘cuticula’ mentioned in the same page of the text, and Tafel XVIII 5–6, respectively)
257 with the cuticle drawn but not mentioned in the captions. Similarly, Kraus (1865) observed
258 the cuticle of *Cycas revoluta*, however provided more precise observations of delicately
259 ornamented cuticles (his Tafel XIX 5–6 and Tafel XX 11), even showing the differences in
260 thickness between the upper and lower cuticles; the term ‘cuticle’ was used about 20 times in
261 this paper. However, Sachs (1873) showed a transversal section of the epidermis of extant *Ilex*
262 *aquifolium* (his Fig. 37) with a thick cuticle made of two parts [outer part labelled ‘a’, devoid
263 of colour according to his text (‘farblos’); inner part labelled ‘b’, with yellow colour (‘gelb’)].

Other authors very rarely figured transversal sections, except for extant reproductive organs (De Saporta 1873–1891); however, De Saporta and Marion (1881–1885) published figures of transversal sections of extant stems (dicotyledons *Nelumbium*, *Tradescantia*, and *Dracaena*) and leaflets (Cycadales *Encephalartos* and *Bowenia* (their Fig. 49–50, 108–109) with clear cuticle differences of thickness between them. Schimper (1874) provided some *Equisetum* transversal sections, with limited details of the outermost part, whereas 10 years later De Bary (1884) used largely transversal sections of cuticles in his figures, some of which were extremely precisely drawn (Figs. 25–27 *Aloe*, *Ilex*, *Pinus*; Fig. 222 *Betula*, Fig. 220 *Ribes*).

Applying the above technique to fossil plants are apparently overlapped in time with extant plant observations, sections of fossil plant cuticles have been documented using light microscopy, and usually with limited details. A few years later after Brongniart's work on extant plants in 1830 and 1834 containing clear descriptions of the cuticle, in the second volume of his fossil plants book, Brongniart (1837) published some transversal sections of permineralized material (petrological thin sections, Pl. 10 Fig. 2 and 3 on lycopods; Pl. 11 Fig. 1 on *Psilotum*), which were apparently influenced by his previous extant plant research: he showed clear epidermis more or less thickened in their outermost parts (as cuticles...), whereas the first volume of this fossil plants book (Brongniart 1828), before his description of cuticles in extant plants, did not contain these details. Renault (1879) followed the same approach with carboniferous material, similarly to 'silicified magma' as he described in the introduction, where *Cordaites* leaves (petrological thin sections, his Plate 16 Figs. 2–7) with epidermis cells were very precisely drawn (using a camera lucida of a light microscope) and clearly different from other types of cells, where the upper epidermis was clearly thicker than the lower epidermis; the cuticle also seemed different in thickness. These very delicate and precise drawings of *Cordaites* were partly reproduced later in several Palaeobotany books

(Potonié 1921, Scott 1923). Some years later, Renault (1893–1896) provided some transversal sections of silicified *Poroxylon incertae sedis* (included in ‘genres à place indéterminée’, *incertae sedis* genera) with clear thickenings over the epidermis (Planche LXXV Figs. 5–6), but without naming ‘cuticle’ in the captions.

These light microscopy section techniques also overlapped with thin sections of organic remnants containing cuticles, according to drawings or descriptions provided, but they were quite difficult to obtain because of their fragility. According to Stace (1965), Brodie (1842) is apparently the first scientist who mentioned the fossil cuticle by distinguishing it from impressions, but did not use the term ‘cuticle’ (‘the epidermis of the fossil frequently peels off... these remains... have been deposited tranquilly between the waters’), which might suggest organic remnants. Among other authors known so far, the term ‘cuticule’ (= cuticle) was used in French by Zeiller in 1882, associated with an extraction method from the sediment to obtain clean material observable with the microscope. He also provided seven precise transversal cuticle sections of different thicknesses and shapes, some of them showing various stomata (his Plate 9 Fig. 8 with *Callitris*, Plate 10 Fig. 14 with *Bothrodendron*, Plate 11 Figs. 7–10 with *Frenelopsis*). De Saporta, in spite of having studied cuticles of extant plants, among his numerous publications, published surprisingly few transversal sections of fossil plant cuticles. However, in 1894 his Plates 5 and 11 contained transversal sections of *Rhizocaulon* roots with a clear outer dark layer of cells, but unfortunately did not include detailed captions, and five sections of *Rhizocaulon* leaves, also with a darker outermost layer of cells like an epidermis (where cuticle is not visible at all). Later on, Zeiller (1900) published a transversal section of *Cordaites* (his Fig. 141), possibly from permineralized material but where the thickening over the epidermis was clear. In 1912, Thompson provided some light microscope photos and drawings of transversal sections of fossil cuticles (his

plates V–VI). In an article with both extant and fossil cycadean plants, Thomas and Bancroft (1913) published some transversal sections of extant cycadean cuticles, which were very clearly drawn in a darker colour than the cell wall (his plates 17–18). Halle (1915) provided transversal sections of the fossils, *Pseudocycas* and *Sciatopitytes* (his Pl. 12 4–6 and 27–29), including a whole leaf section. Seward's book on fossil plants (1919) contained a transversal section of *Nilssonia brevis* (his Fig. 620 B after Nathorst), which was unfortunately not detailed, whereas that of *Ctenis* sp. (his Fig. 625 B) had a clear darkened cuticle above the epidermis. Potonié (1921) published some transversal sections of a *Nilssonia* leaf (his Fig. 234 c), with a very homogeneous cuticle and devoid of cell remnants. Florin (1933, 1936) provided fossil transversal sections ('Querschnitte') of cuticles of Ginkgoales (his Tab. 8 and 20) and Cycadales (from Tab. 2 to Tab. 13).

Following this period of sections in light microscopy through the XXth century (see Stace 1965 for a detailed chronology of cuticle findings), the first fossil cuticle sections of material embedded in resin and observed using ultrastructural TEM began in 1986 with the genera *Tarphyderma* and *Ticoa* (Archangelsky and Taylor 1986; Archangelsky et al. 1986), after TEM images of Miocene leaves (angiosperm *Zelkova*, Niklas et al. 1978; see also Fig. 1. 24 of Taylor et al. 2009) were published, where the 'cuticular membrane' appeared to be illustrated (their Fig. I, K), but was not described in detail.

1.2. Various aspects of plant cuticle ultrastructure

These fossil studies contained several important topics and contents quite classical in palaeobotany that emerged from the beginning of this research and are still developing. The first topic discussed in papers from 1986 concerned taxonomy. Although Holloway (1982)

proposed six types of cuticles for living plants, grouping very different taxa in each type [from *Selaginella* to angiosperms] from different parts of plants (leaves, fruits, ovules, stems, trichomes, stigma...), he was very possibly conscious of the importance of cuticles in taxonomy as he concluded: ‘Because the CM of plants is so heterogeneous in structure it is dangerous to oversimplify and generalize about its morphology and construction – there is no typical CM. Consequently, each species must be considered individually...’ Although in living plants, until recently, TEM details of cuticles have seldom been provided, they are largely provided in fossil plant taxonomical studies, and still prove that 1) one cuticle may refer to several types of those described by Holloway (1982) and many overlaps occur, and therefore the six described types are not enough; 2) in one single type each species has its own identity with specificities in thicknesses and/or proportions of layers and/or sub-layers. In a study on *Ticoa*, Archangelsky et al. (1986) stated that ‘the structural and perhaps histochemical complexities of fossil plant cuticles will require some further division of these layers’, enhancing all the future detailed taxonomical articles developed.

Palaeoenvironment is the second topic discussed in term of number of papers. This topic has been developed regularly widely in various fossil plant groups (Kerp and Barthel 1993, Krings and Kerp 1997). More precisely for TEM, in a study on *Tarphyderma*, Archangelsky and Taylor (1986) suggested ‘...additional insights into ecological parameters...’, ‘...response to the habitat...’, and ‘...various structural features were evolving in other major groups of plants in response to the habitat...’ Additionally, Archangelsky et al. (1986) pointed out that “... the presence or absence of specific cuticle layers in the fossils may be directly associated with the environment in which the plant was living...”

The third topic that has been up to now very rarely documented, concerns evolutionary aspects. In the *Ticoa* study (Archangelsky et al. 1986), when emphasized the importance comparisons between fossil and living plants cuticles, these authors pointed out comparisons between fossil and living plants cuticles ('subsequent work with fossil plant cuticle ultrastructure will consider both the taxonomic application and evolutionary significance of these features'. In a study on *Tarphyderma*, Archangelsky and Taylor (1986), suggested 'adaptations' and more precisely 'structural adaptations in fossil plants'.

Two other points have not been included in this review, as they have not been documented in detail. The first concerns ontogeny, which is classical if working on features of organisms, evocated also by Archangelsky et al. (1986), who suggested that "...the presence or absence of specific cuticle layers in the fossils... may reflect a particular level of organ development at the time of fossilisation". This has been studied in living plants (*Sphagnum*, moss, Jeffree 2006; algae, Jeffree 2006; *Picea*, gymnosperm, Jeffree 2006; *Hedera helix* L., angiosperm, Viougeas et al. 1995; *Utricularia*, angiosperm, Jeffree 2006; *Solanum lycopersicum*, angiosperm, Dominguez et al. 2011b and 2012, Segado et al. 2016; *Arabidopsis*, angiosperm, Nawrath 2006, Fabre et al. 2016; see also Fernandez et al. 2016; see also the reviews of Yeats and Rose 2013 and Ingram and Nawrath 2017); however, until now, no fossil studies have been carried out to the best of our knowledge in this regard, owing to the extreme rarity of different stages of the same material found in the fossil record. The second concerns diagenesis and TEM, carried out from 1986 (Archangelsky et al. 1986). Although this is fundamental for the study of fossil plants, the analysis is difficult to carry out. At least within one genus, TEM data comparing fossil (170 millions of years) and living ginkgos, Guignard and Zhou (2005) found very few differences in the cuticles within this genus, although there was a considerably minimised effect of diagenesis.

2 Methods

The details of the methods used to obtain the sections of cuticles are somewhat variable, related to different chemicals used, also depending on the material (preservation and others...) and taxa, the technique of the laboratories.. However, the methods for this especially difficult preparation (Taylor 1999, in a special chapter on the ultrastructure of fossil cuticles) followed a similar general scheme as summarised here. According to all the studies selected in Table 1 where the main differences in methods are listed, the material could be extracted from the sediment treatments (or not) with several chemicals during variable times. Moreover, the removal of minerals and sediments if necessary (in order to not destroy the glass or diamond knife when cutting the sections) is also summarised: hydrofluoric acid, followed by then Schulze's reagent (nitric acid and potassium chlorate; Gray 1965, Traverse 1988), and finally dilute ammonia. Although the same method should be used for effective comparisons between cuticles, the use of these treatments produced very damaged areas in some cuticles (holes, breakings, and missing parts), not allowing detailed observations, especially collecting of enough statistic data possible. In some cases, untreated cuticles provided much longer areas of entire cuticles, which were very comparable with treated material, and are more suitable for observations and photography. Apparently no (or very few) changes are noted in all taxa where it is used (Guignard and Zhou 2005, Del Fueyo et al. 2013, Guignard et al. 2016, Yang et al. 2018, Carrizo et al. 2019a, this issue; Guignard et al. 2019, this issue).

TEM methods (Appendix A of MethodX article) begin (or not) with the fixation of cuticles (in glutaraldehyde or paraformaldehyde), then a postfixation (or not) in Osmium tetroxide,

413 followed by dehydration in degraded solutions of alcohol allied with graded percentages of
414 resin for its impregnation in the cuticle (Spurr, Epon, Araldite). Finally, a polymerisation of
415 the blocks of resin (at 37, 56, 60 or 70 °C) is achieved depending on the type of resin, during
416 variable times. The majority of research referred to only one kind of resin for each paper, this
417 homogeneity allows comparisons, since some differences are found with various uses of resin
418 for the same taxon (extant *Eucalyptus*, *Populus*, *Pyrus*; Guzman et al. 2014).

419 From this step, two kinds of sections, using an ultramicrotome, can be obtained from the
420 blocks of resin embedding the cuticles (Appendix B of MethodsX article), usually cut with a
421 diamond knife, but more rarely with a glass knife: 1) rarely mentioned but very useful are thin
422 sections (1 µm) mounted on glass slides and stained (in blue colour) using the methods of
423 Richardson et al. (1960), observed with a light microscope, enabling to the quality of the
424 cuticle to be analysed and determine the best sections with the maximum data (containing not
425 only ordinary epidermal cell cuticle, but also potentially stomata and the base of trichomes
426 and papillae); 2) ultrathin sections of 60–70 nm (silver to pale gold), observed using TEM,
427 deposited on uncoated or Formvar-coated 200–300 Mesh copper grids (or other meshes, other
428 metals, even with one slot grids in rare cases). The majority of sections are transversal to the
429 cuticle (i.e. perpendicular to the leaf length), but some are longitudinal (i.e. parallel to the leaf
430 length) to illustrate the homogeneity of the material through these two orientations.

431 The last step is staining, to obtain high contrasts of the different materials comprising the
432 cuticles. This step shows high variations of techniques, not only in the chemicals (potassium
433 permanganate, uranyl acetate, lead citrate), but especially in combinations of durations and
434 dilutions. Although most papers use only one method, some articles refer to several attempts
435 for the same material: in fossil gymnosperms, Taylor et al. (1989) provided TEM photos of
436 cuticle with different staining times (60-20-10 minutes, or 25-25-17, or 20-20-10) used for the
437 three chemicals, respectively. In Umkomasiales (Labe and Barale 1996), the staining was

adjusted (in times and dilutions) according to the material. In Czekanowskiales (Zhou and Guignard 1998), five methods of staining were used with the three chemicals (or two or even just one), with various times and percentages of solutions. Their best staining is indicated with uranyl acetate (20 min) and lead citrate (20 min). As the type of molecules stained in TEM is questionable by some authors, through the photos provided in the papers for one taxon (= same type of material), apparently it is difficult to state if different molecules are stained with different stains; only lower or higher contrasts seem to be relevant, but exercising caution is recommended.

As an example, the technique of Lugardon (1971) is provided here (Appendix A of MethodsX article), enabling very clean and highly contrasted details of cuticle sections. This technique was formerly used for spores (living and fossil plants) with Spurr and/or Epon resin (as in *Marattia* spores with Lugardon as a co-author, Wang et al. 2001), and adapted for cuticles with Epon resin. Compared with other embedding techniques of 1–2 days very common for TEM studies of living organisms, this one is particularly long (one week of procedure, from day 1 to day 8), which is considered very useful for a low risk of breakings of the cuticle when obtaining thin or ultra-thin sections. Osmium tetroxide postfixation and staining with both uranyl acetate and lead citrate (Appendix B of MethodsX article) allow a very satisfying contrast of the contents of cuticles, enabling high magnifications > 100,000 ×.

In this review, the methods of the three first aspects (taxonomy, palaeoenvironment, evolution) were carried out, and multifactorial analyses were applied, using XLSTAT 2019.1 and XLSTAT3D Plot (Addinsoft (2019); XLSTAT statistical and data analysis solution. Long Island, NY, USA. <https://www.xlstat.com>.), for chi-square automatic interaction detector (CHAID), factorial component analysis (FCA), and hierarchical components (HCA).

3 Major results and discussion

3.1 Significances and application in fossil plant taxonomy

3.1.1. Detailed description of different parts of fossil plant cuticles.

Although in living plants mechanical, physical and chemical aspects are searched extensively (Dominguez et al. 2015, and 2017 with more than ten articles of a special issue entitled “The plant cuticle, old challenges, new perspectives”), allowing Cohen et al. (2017) to write that “A search in Web of Science... shows that the number of published articles associated with the cuticle gradually increased each decade starting in the 1950s up until the late 1990s...”, detailed description of the different layers of the cuticle is seldom given. However they do exist as in living *Ficus elastica* (Guzmán-Delgado et al. 2015), in *Arabidopsis thaliana* (Mazurek et al. 2017, Berhin et al. 2019), in orchids (*Bulbophyllum weberi* and *B. cumingii*; Kowalkowska et al. 2017), in tea (*Camellia sinensis* cv Fuyun; Zhu et al. 2018 and *Opuntia* (Agüero et al. 2018). In fossil cuticles, mainly leaf cuticles being reviewed here [although cuticles are also present in fruits, stems, flowers and embryos as revealed in living plants (Heredia 2003, Heredia-Guerrero et al. 2017, Ingram and Nawrath 2017), all these organs being very fragile for preservation], descriptions are very frequent, possibly because cuticles are often the only remains of fossil plants. The description of the fossil cuticle (= cuticular membrane CM) was first proposed by Archangelsky et al. (1986), following the scheme of Holloway (1982) for living plant cuticles, who summarised detailed cuticle sections from von Mohl’s description (1847; see particularly the distinct Figure 4 in this 1847 volume, showing two clearly distinguished parts of the cuticle, above the cell wall; see also von Mohl 1842, Geneau de Lamarlière 1906, Roelofsen 1952 for his photos and schemes, Roelofsen 1959 for

his Fig. 167). This scheme (Fig. 1 and Plate I for illustrations) with two parts in a fossil cuticle is still used, completed by sublayers of A1 and A2, following the studies of Guignard and Zhou (2005) and Nosova et al. (2016). This scheme is not as definite as that of Holloway (1982), for extant plants, "...it is always inadvisable to present a single generalised scheme for the construction of the plant CM". The cuticular membrane (appendix A) is divided into two regions: an outer cuticle proper (= CP = A) directly in contact with the atmosphere in its outermost part, and an inner cuticular layer (= CL = B) in contact with the cell wall of the subjacent cell (mostly absent in fossil material) in its innermost part. The outer cuticle proper A can be divided in two layers: an outermost A1 polylamellate layer subdivided in some cases into A1U (= upper zone or sublayer, which can be straight or wavy) and A1L (= lower zone or sublayer), then an inner A2 granular layer, divided in some cases into A2U (= upper zone or sublayer) and A2L (lower zone or sublayer). The inner cuticular B can be divided in two layers: fibrilous or spongy B1 in its outer part and granular B2 in its innermost part. This scheme is very synthetic and generalized, and some taxa may have only part of these layers and /or sublayers.

3.1.2. At the level of order and/or family: qualitative descriptions

According to the presence-absence of layers and/or sublayers (zones) of cuticles, which are variable in fossil plants studied to date (mainly leaf cuticles), for the ordinary epidermal cuticle cell a simple formula can be proposed, from the simplest one (A2) to complex ones (for instance A1U + A1L + A2U + A2L + B1). Two syntheses are proposed here, one with the presence or absence of A1, A2, B1, B2, selected successively (Fig. 2), one with an HCA developed by software using the combination of all these characters (Fig. 3). In the potential ultrastructural dichotomous key developed 'manually' (Fig. 2), in some cases, the words 'wavy, spongy, fibrilous' were added to this formula to enable the distinction from other

cuticles. Moreover, it is noticeable that although the number of combinations of layers and sublayers is relatively high, the actual number of cuticle formulae is relatively low (11) and corresponds to taxonomic groups, where two kinds of groups occur: homogeneous and heterogeneous groups, making this formula an excellent tool for taxonomy.

As a result of Fig. 2, in homogeneous groups (5), each order (or family) contains genera with only one cuticle formula: Coniferales Cheirolepidiaceae, comprising *Pseudofrenelopsis-Suturovagina-Hirmeriella*, all with a wavy A1-A2-B1-B2 formula; Coniferales Miroviaceae, comprising *Mirovia*, with a A1U-(A1L)-A2U-(A2L)-B1 formula; Coniferales Araucariaceae, comprising *Nothoprehuen-Araucaria-Brachyphyllum*, all with a A2-spongy B1 formula; Caytoniales, comprising *Rufloiria*, with a A2-B1-B2 formula different between upper and lower cuticles; Czekanowskiales comprising *Arctobaiera-Phoenicopsis*, also with A2-B1-B2 formula different between upper and lower cuticles.

In heterogeneous groups (3), each order contains two groups of formulae (making a total of six): Ginkgoales, comprising *Baiera-Sphenobaiera* with A2-B1 (type I) and *Ginkgo-Ginkgoites-Pseudotorellia* (type II) with A1U-A1L-A2-B1; Pteridospermales, comprising *Dichopteris-Komlopteris* with A2 (type I), and *Pachypteris* with A1-A2-B1 (type II) showing differences between upper and lower cuticles; Cycadales comprising *Pseudoctenis-Ticoa* (type I) with A1-A2-B1 and *Restrepophyllum* with A1-B2 (type II). If the ultrastructural unity of cuticles of one order (or family) is reinforced if they have just one cuticle formula, for the separation of an order in two ultrastructural cuticle groups the relationship between cuticle ultrastructure and gross morphology is unclear. Actually, even with two groups it makes such heterogeneity taxonomically inconclusive and it would be effective to increase the number of taxa to accurately determine these formulae. For instance, in Ginkgoales (Guignard et al.

2019, this issue), the authors relate that *Ginkgo* and *Ginkgoites* are relatively close in gross morphology (which suits their ultrastructural affinities), while *Pseudotorellia* is different in gross morphology, however the three taxa have the same cuticle ultrastructural formula. It is also the case for *Baiera* which is different from *Sphenobaiera* in gross morphology, but they have the same cuticle ultrastructural formula.

Using the HCA of the same data and calculated by using data 0–1 (Fig. 3; Appendix A of data in brief article), 52 ordinary epidermal cell cuticles from more than 25 taxa have been considered. As these cuticles are considered individually when lower and upper cuticles are available for one taxon, if their cuticle formula is different in one single taxon, it makes some separations in these taxa. However, the identification of orders-families is similar to Fig. 2. This combination of the presence-absence of layers and sublayers by HCA resulted in 9 groups (instead of 11 in Fig. 2; the two ‘missing’ groups corresponded to 2 orders allied with 5 other cuticles from other orders), namely: Cycadales type II, Coniferales (Cheirolepidiaceae, Miroviaceae, Araucariaceae), Ginkgoales type I and II, Pteridospermales type I and II, Czekanowskiales and Cycadales type I, plus some other cuticles of the former groups.

So far as the former studies revealed where the stomatal apparatus could be observed, subsidiary cell cuticles are usually similar to ordinary epidermal cell cuticles in ultrastructure and give the same formula classification. It is not the case for guard cell cuticles, which are usually different from the cuticle of ordinary epidermal cells and subsidiary cells. Although they are rarely observed, owing to their fragility, the guard cell cuticle is of great taxonomic importance as a multidimensional CHAID classification of the taxa could be also proposed. (Fig. 4; Appendix B), using another order of characters than for ordinary epidermal cell

cuticles formerly developed in this paper (Fig. 2 with the following order: A1 or not A1, A2 or not A2, B1 or not B1, B2 or not B2). For guard cell cuticles, firstly the cuticle proper A is involved in the presence/absence of the A1U sublayer, then two possibilities occur (presence/or not of the A2 layer, or presence/or not of the A1L sublayer). The cuticular layer B is then involved, with the presence/or not of B1 and B2 layers. In the few taxa where cuticle guard cell details could be observed, Coniferales (Araucariaceae, Cheirolepidiaceae; Carrizo et al. 2019a this issue, Mairot et al. 2014, Yang et al. 2018, Yang et al. 2009, Guignard et al. 2017), and Pteridospermales (Guignard et al. 2004, Thévenard et al. 2005) only one type of guard cell cuticle formula can be distinguished. Ginkgoales (Guignard and Zhou 2005, Guignard et al. 2016, Del Fueyo et al. 2013, Wang et al. 2005, Guignard et al. 2019 this issue) showed two possibilities (group 1 with A2 and B1 layers, group 2 with A1U and A1L, A2, B1), and it is noticeable that two groups could be divided as those with ordinary epidermal cell cuticles OEC (*Baiera* and *Sphenobaiera*, *Ginkgo-Ginkgoites-Pseudotorellia*) (Guignard et al. 2019, this issue). As only one taxon (*Ginkgoites skottsbergii* which is referred to a form genus of Ginkgoales) does not follow this scheme (it is grouped with *Baiera* and *Sphenobaiera*), attention should be given to guard cell cuticles in the future.

3.1.3. At the level of the genus and species: quantitative description and statistical values

Cuticle ultrastructures and measurements are related to the creation of a taxon, its description, and distinction from related taxa where, generally, values are largely involved. According to the International Code of Nomenclature for Algae, Fungi, and Plants [2018 Shenzhen; see also the IOP International organization of Palaeobotany: <https://www.palaeobotany.org/page/nomenclature/nomenclature-typification-and-correct-description-of-fossil-plants>, points 2. Correct description of new (fossil) plant taxa (genera, species) and new combinations 2.1 General rules], a new taxon name must be accompanied

by a description or diagnosis (or by a reference to a previously published description or diagnosis) (Art. 32.1d), a diagnosis of a taxon being ‘a statement of that which in the opinion of its author distinguishes the taxon from other taxa’ (Art. 32.2). In the ‘recommendations’, three points are of great interest as they state the level of importance attributed to the characters used for a new taxon: 1) the diagnosis should be concise and based on the most important characters only; 2) the description should include all additional data of secondary importance (i.e. not included in the diagnosis), such as measurements; 3) a discussion with comparison to other species; this should provide the differential characters in which the new species differs from other species, at least in the same genus. Furthermore, a fourth point should be added to these three formal points: in Palaeobotany the use of diagnosis emendation also occurs, corresponding (in the case of cuticle ultrastructure) to a change of definition thanks to newly observed characters compared with the original ‘older’ diagnosis, related to the use of new methods of study.

Although there are different opinions as regard to the taxonomic value of fossil plant cuticle ultrastructural features, which may include simple evaluations of layer and sublayer thickness, their percentages, to more elaborate measurements with statistical evaluations (confidence interval and other tests) based on a high number of values (up to 30 so far known in this review), recent studies have become more and more clearly shown that the ultrastructural features of fossil cuticles may be considered as significant in taxonomy, as any other character.

First, cuticle ultrastructure characters were included in the diagnosis with the creation of the genus *Squamastrobis* by Archangelsky and Del Fueyo (1989). After that, such important characters have been given in the diagnosis of two conifers (*Morenoa fertilis*, Del Fueyo et al. 1990; *Glenrosa nanjinensis*, Zhou et al. 2000), a new Araucariaceae taxon (*Nothopehuen*

brevis; Del Fueyo 1991) or with araucarian affinity (*Brachyphyllum garciarum*, Carrizo et al, 2019 this issue), three new cycads (*Pseudectenis ornata*, Archangelsky et al. 1995; *Mesosingeria oblonga* and *Ticoa lanceolata*, Villar de Seoane 2005), three new Bennettitales (*Otozamites ornatus*, Villar de Seoane 1999; *Otozamites patagonicus*, Villar de Seoane 2001; *Ptilophyllum eminelidarum* Carrizo et al. 2019b), and one Pteridospermatophyta (*Ruflorinia orlandoi*; Carrizo et al. 2014). Not only cuticle ultrastructure characters of vegetative organs, but also those of the reproductive material (megaspore membranes) have also been incorporated into the diagnosis (*Karkeniania* of Ginkgoales, Zhou et al. 2002; Del Fueyo and Archangelsky, 2001).

Second, the description of a new taxon: a new genus (*Tarphyderma*; Archangelsky and Taylor 1986) was created, including after the diagnosis a description of the ‘cuticle ultrastructure’. Artabe et al. (1991) included the cuticle ultrastructure of *Kurtziana brandmayri* in their description; the description was used in 1992 for ‘complementary details’ of *Squamastrobis tigrensis* (Barale et al. 1992); for a new combination (*Pachypteris bagualensis*) a cuticle ultrastructural description was included (Baldoni and Barale 1996); Passalia et al. (2010), created a new genus with a new species (*Restrepophyllum chiguoides*), and the cuticle ultrastructure was included in their ‘description of the leaf cuticular membrane ultrastructure’. Description was also detailed with three species of Miroviaceae (Coniferales; Nosova et al. 2016). Connected with the diagnosis of *Ptilophyllum eminelidarum* (Carrizo et al. 2019b), the authors also provided a precise description of the layers of the cuticles.

Although many other papers do not include fine ultrastructural details in the definition of a taxon, these papers enhance their importance for taxonomy in the ‘results’ or ‘discussion’ sections. This was the case for two Czekanowskiales (Zhou and Guignard 1998), with details

of their affinities and specificities. For *Pachypteris indica* and *Komlopteris indica* (Bajpai and Maheshwari, 2000), the ultrastructure was described in ‘observations’ and ‘comparisons’ sections. For *Dichopteris* (Pteridosperm; Thévenard et al. 2005), the ‘cuticle microscopy’ occurred in the ‘descriptions’ section, whereas it was placed in ‘results’ section for *Sphenobaiera huangii* (Ginkgoales; Wang et al. 2005). This was also the case for *Pachypteris* (Guignard et al., 2004), *Pseudofrenelopsis*, and *Hirmeriella* (Cheirolepidiaceae, Guignard et al. 1998, Yang et al. 2009), and *Ginkgoites* (Ginkgoales, Del Fueyo et al. 2013). Reproductive material is also relevant in this field (fertile Podocarpaceae, Archangelsky and Del Fueyo 1987; corystospermaceous ovules, Barale et al. 2009).

Third, the use of ultrastructural comparisons between several taxa of the same group also exist in single papers, with specificities and/or affinities enhanced, at various taxonomic levels. Barale and Baldoni (1993) compared the ultrastructure of five species of Bennettitales; for four Umkomasiales (Labe and Barale 1996), two species belonged to the same genus (*Pachypteris*); taxonomy was then developed in their discussions, and the homogeneity at the order level was enhanced, as well as specificities. Specificities within one genus were also developed between two species of *Dicroidium* (Corystospermaceae) (Maheswari and Bajpai, 1996a). In 2003, descriptions of six Bennettitales taxa were published, including TEM details, and three ultrastructural groups were proposed (Villar de Seoane 2003).

In some studies, comparisons with living taxa were developed. Early in 1986, the *Ticoa harrisii* cuticle ultrastructure characters were included in a description of this taxon and compared with those of extant plants, mainly following the six types of Holloway (1982) (Archangelsky et al. 1986). In 1989, two fossil cuticles (*Ginkgoites tigrensis* and *Karinopteris*) were described and compared with the cuticle of living *Ginkgo biloba* (Taylor

et al. 1989). This living taxon was also compared with *Ginkgoites tigrensis* (Villar de Seoane 1997) and fossil *Ginkgo yimaensis* (Guignard and Zhou 2005). Similarly, *Mesodescolea plicata* (fossil Cycadales) was compared with the living *Stangeria paradoxa* (Artabe and Archangelsky 1992), and *Restrepophyllum chiguoides* (fossil Cycadales) was compared with living *Zamia* (Passalia et al. 2010).

Fourth, an emendation of the diagnosis of *Ginkgoites tigrensis* was published, immediately before the description, with the cuticle ultrastructure (Villar de Seoane 1997). The following year, *Tomaxellia biforme* (Cheirolepidiaceae) and *Athrotaxis ungeri* (Taxodiaceae) were compared with two living Taxodiaceae, and an emended diagnosis including ultrastructure was proposed (Villar de Seoane 1998). This was also the case for *Araucaria grandifolia* (Araucariaceae; Del Fueyo and Archangelsky 2002), *Ptilophyllum valvatum* and *Cycadolepis coriacea* (Bennettitales; Villar de Seoane 2001), and the new combination of *Ginkgoites patagonica* with an ‘emended species diagnosis’ (Villar de seoane et al. 2015).

Based on data of 41 fossil cuticles studied with TEM, of which absolute values and percentage of characters (23 in total) could be analyzed [selected from the 52 cuticles used in the 0-1 data matrix (Appendices A and B of data in brief article), and their curve tendencies (Appendix C), the correlations between some of them (26) are simple and linear ($y = ax + b$) for about 33% (8 of them: CM mean – A2 mean, A1U straight mean – A2L mean, A2L mean – B1 fibrilous mean, A2U mean – B2 mean, OL mean - TL mean, A% - B%, A2U % - B1 fibrilous %, A2L % - B1 fibrilous %), and the remaining cuticles (18) having complex and polynomial correlations (even at the order 6 for A1L % - A2U %, CM mean – A1U straight mean). This is a demonstration of the complexity of the relations between cuticle characters as shown by their fine details. In only 33% of the 41 cuticles, one character can be deduced

easily from another one, so although a part of the details are very simply (in mean and %) correlated, most of them have very complex relations. The FCA developed with these 41 cuticles and 23 characters (Figs. 5 and 6; Appendix B of data in brief article) showed that, with a very satisfying 93.54% of information on the three first axes (1, 2, 3), six main characters are involved in the distinction between the taxa, i.e. firstly the cuticular layer B (Fig. 5; B %, fibrilous B1%, B2 mean), then the cuticle proper (A % and A2U %), and finally the total mean of the cuticular membrane (characters numbers in Fig. 5: 17, 21–22, 3, 13, 1). At a much lower degree of importance, some other characters are relevant: A mean, A2U mean, A2L mean and % of the cuticle proper; B mean, fibrilous B1 mean, B2% of the cuticular layer (characters numbers in Fig. 5: 2, 23, 14–15, 16, 12, 20). As compared with the former characters, none of the eight A1 characters of the cuticle proper (numbered 4–11 in the figure) showed importance for the distinction between cuticles in this FCA. Concerning the distinction between cuticles (disposed according the absolute values and % of characters; Fig. 6), all groups of the HCA (made with qualitative 0-1 presence-absence; Fig. 3) are also relevant (except Cycadales type II, which could not be considered in this FCA): Coniferales (Cheirolepidiaceae, Miroviaceae, Araucariaceae), Ginkgoales type I and II, Pteridospermales type I and II, and Czekanowskiales. For some groups, the distinction is still clear (Coniferales Araucariaceae, Pteridospermales type I, Ginkgoales type I) and explained by the homogeneity and small variability of the absolute values and % of thickness of the constituents in each group. However, this FCA is fundamental as the values and % of the cuticles of the other groups are much more heterogeneous, demonstrating a higher variability, and the positions of the cuticles are finer and inter-linked (especially Ginkgoales type II and Coniferales Cheirolepidiaceae, at a lesser degree Coniferales Miroviaceae, Czekanowskiales, Pteridospermales type II), revealing some possible affinities between these groups.

714 The cuticles of Cheirolepidiaceae have been studied in detail with TEM [showing 4 types of
715 cuticles: two *Suturovagina intermedia*, one from less and the other from more xerothermic
716 palaeoenvironments (Mairot et al. 2014, Yang et al. 2018); and two *Pseudofrenelopsis* species
717 also from Cretaceous of China (Yang et al. 2009, Guignard et al. 2017)], multidimensional
718 analyses revealed to be a good taxonomic tool and various points could be enhanced
719 (Appendix D). First, the correlations between the curve tendencies of all Cheirolepidiaceae
720 cuticle characters (Appendix E), are different and more particular to this family than those of
721 all 41 cuticles from various families: for these Cheirolepidiaceae cuticles, among 17
722 correlations, they were simple and linear ($y = ax + b$) only for 3 of them (A2 mean - B2 mean,
723 A% - B%, A2% - B1%), most of them having complex and polynomial correlations (even at
724 the order 6 for A1% - A2%, OL mean - TL mean, A1 mean - A2 mean, CM mean - A1
725 mean). This is a demonstration of the peculiarities of this family (in comparison, the curve
726 tendencies made with all four types of cuticles show eight linear correlations; Appendix C) in
727 which one cuticle character cannot be deduced very easily from another one, except for very
728 few of them (16.7%), and though each cuticle has its own equilibrium of details (in mean and
729 %), enhancing the interest of very delicate observations in this family. Second, from the FCA
730 developed with nine cheirolepidiacean cuticles (ordinary epidermal cell and subsidiary cell
731 cuticles; 99.44% of information on the first 3 axes; Fig. 7; Appendix F), the importance of 15
732 characters (Fig. 7a) was ordered as follows: percentages are more important for the
733 construction of the first and main axis 1 (especially A% and A2% of the cuticle proper, B%
734 and B1% of the cuticular layer, all with more than 0.2 of contribution on axis 1), whereas
735 means (except CM mean for axis 2) and other percentages are less or not important. Third, the
736 thickness mean of opaque or translucent lamellae of the A1 wavy layer is not important at all
737 for the construction of the axes (contribution of 0 on axis 1). Concerning the relative positions
738 of the nine cuticles, except one (subsidiary cell cuticle of less xerothermic *Suturovagina*

739 *intermedia*), each of the four types was identified relatively clearly by HAC (Fig. 8).
740 Similarly, for the FCA drawn with the ellipse confidences (Fig. 7b), which was more subtle in
741 the relations between the cuticles as some of them had a higher variability in their absolute
742 values and % than did the others. It is interesting to note that confirming the originality of
743 guard cell cuticles GC discussed formerly, the addition in these multidimensional analyses of
744 three guard cell cuticles (HCA with 12 cuticles; Fig. 8b) increased the heterogeneity of the
745 scheme slightly as guard and subsidiary cell cuticles of less xerothermic *S. intermedia* are
746 more clearly separated from the ordinary epidermal cell cuticles of the same type of cuticles
747 and make then a stronger group. This was also true for the FCA with 12 cuticles (with added
748 GC) with slightly less information than the FCA with 9 cuticles (95.22% of information on
749 the 3 first axes; Appendix G), where the weight of the characters inducing the axes was also
750 different: the cuticle proper was the most informative for axis 1 (50.5% of information) with
751 B and B1 means, B% and B1%; the cuticular layer contributed to axis 2 (34.46% of
752 information) with A and A2 means, A1%.

753
754 Recently, Energy Dispersive Spectroscopy (EDS) element analyses were added to
755 ultrastructural features and included in the taxonomic value: *Ginkgoites* (Ginkgoales,
756 Guignard et al. 2016, Del Fueyo et al. 2013), *Suturovagina* and *Pseudofrenelopsis*
757 (Cheirolepidiaceae, Guignard et al. 2017, Yang et al. 2018), *Baiera* and *Sphenobaiera* of
758 Ginkgoales (Guignard et al. 2019 this issue), *Brachyphyllum* of Araucariaceae affinity
759 (Carrizo et al. 2019a, this issue).

762 3.2 Palaeoenvironmental significances

763 Cuticles have been increasingly intensively studied for various relations with the environment

in living plants [Dominguez et al. 2011a and b, 2017; Martin and rose 2014; Khanal and Knoche 2017; Schuster et al. 2017; He et al. 2018; Heredia-Guerrero et al. 2018; particularly in *Arabidopsis*, Kosma et al. 2009 (cited in Heredia-Guerrero et al. 2017); in pear fruit *Pyrus communis* L., Serra et al. 2018; in eggplant (*Solanum melongena* L.), Valerga et al. 2019; in sunflower (*Helianthus annuus*), Li et al. 2019; in two *Leucospermum* cultivars (Proteaceae), Suarez et al. 2019; also including (Aragón et al. 2017) in their review of many papers, external and internal environmental factors like fungi, bacteria, viruses, yeasts and insects (Gorb and Gorb 2017) and herbivorous]. From the beginning of TEM fossil cuticle studies, the putative influence of the environment on cuticles has been envisaged; however, at different levels and various degrees of precision. In their TEM study of *Tarphyderma*, Archangelsky and Taylor (1986) discussed ‘xeromorphic features’; however, unfortunately did not include high enlargements. In the same year, Archangelsky et al. (1986), in *Ticoa*, considered the ‘A2 sublayer’ could have ‘some ecological significance’. Taylor et al. (1989) suggested that ‘the overall cuticle thickness differences could be attributed to a number of factors, the most probable of which are ecological...’ in their *Ginkgo* study. Moreover, they began to enhance fine details to determine the possible significance of the lucent lamellae thickness of the A1 layer, evocating ‘ecological’ significance among other potential interactions. Archangelsky (1991) discussed the interest of ultrastructural studies of fossil cuticles in strata of different ages and varied ‘palaeoecological settings’, but unfortunately did not provide details.

For the first time, Barale and Baldoni (1993), reported in their TEM study on the cuticle ultrastructure of a bennettitalean fossil, a possible relation between cuticle details (their Fig. 8) and xerophytism; however, it did not directly concern the whole cuticle but a zone between the polylamellate layer of the cuticle and outermost wax material. Several authors have

carried out similar studies: Maheswari and Bajpai (1996b) reported the biochemical degradation of the cuticle of *Thinnfeldia* owing to fungous contamination using TEM analysis. In the TEM study of *Cycas circinalis* (Bajpai, 2000), the possible influence of the environment on the ‘outer polylamellate and the inner reticulate zones’ of the material was discussed. For *Pachypteris indica*, Bajpai and Maheswari (2000) suggested the possible ‘definite’ influence of ‘ecological conditions’. Villar de Seoane (2001) noted that the TEM cuticle details of some Bennettitales (with ‘a compact and uniform layer, and a thick lower layer that may be further divided in two sub-layers’) could be related to the volcanic activity). Passalia et al. (2010) stated that “regardless the cuticular membranes thickness, most of them were adapted to low rates of transpiration because they have an outer lamellate layer...” in studying a fossil cycad (*Restrepophyllum*).

With the increasing number of studies, comparisons among genera of the same taxa (family or orders) began to emerge and provided higher degrees of precision about the influence of the environment on the cuticle details. These kinds of comparisons had been clearly enhanced early (*Tarphyderma* study; Archangelsky and Taylor, 1986), where the authors suggested to “...compare epidermal and cuticular features from similar taxa that grew in different environments...” The studies of two groups can be evoked here. The first group is Cheirolepidiaceae (Coniferales). All the following cheirolepidiaceans studied in ultrastructure: *Hirmeriella muensteri* (Guignard et al. 1998); *Pseudofrenelopsis dalatzensis* (Yang et al. 2009); *Suturovagina intermedia* (Mairot et al. 2014) and Yang et al. (2018); *Pseudofrenelopsis gansuensis* (Guignard et al. 2017) exhibited a number of xeromorphic characters, not only a thick cuticle, the presence of a cutinized hypodermis, and other ‘morphological characters that prevent excessive evaporation from the leaves’; but also fine details, including a thick and wavy polylamellate zone (A1 layer) with measurements of opaque and translucent lamellae at

the nanometer scale. The second group of taxa with studied cuticles concerns Ginkgoales, where several species and genera were compared. *Ginkgoites ticoensis* (Del Fueyo et al. 2013; their Fig. 4A–4C), showed fibrils of the B1 cuticular layer becoming parallel to the cell surface, interpreted as the volcanic environment effect, as a result of comparisons with living *Pinus* cuticles in the same environment. This was also the case for electron-transparent areas (their Figs. 4E, 4F, 7H) in the cuticle, related with oxalate calcium crystal deposits of volcanic area in the same living *Pinus* material. A last aspect does not directly consider cuticle details, but ‘epicuticular and epistomatal waxes’, which seem to ‘undergo fusion as they are visible in *G. tigrensis* (Villar de Seoane 1997; their Pl. 1, Fig. 5, Pl. 2, Fig. 1)’, also from volcanic environments. In *Ginkgo*, conversely to *Ginkgoites*, the potential effect of the environment on the leaf cuticle ultrastructure seems to be not sensitive, as comparisons between living *G. biloba* and the oldest species *G. yimaensis* in China (Lower Middle Jurassic) concluded that ‘the consistent leaf cuticle ultrastructure of both fossil and living ginkgos indicates that environmental conditions have also little effect’ (Guignard and Zhou 2005). In *Baiera* and *Sphenobaiera* (Guignard et al. 2019, this issue; see also Wang et al. 2005), although palaeoenvironments were warm and humid for both taxa, *Baiera* (with a thinner cuticle) possibly vegetated under a slightly cooler climate than *Sphenobaiera* (with a more than twice thicker cuticle); this is in congruence with associated flora and EDS elements of the cuticle. The occurrence of a warm and humid, palaeoenvironment (Santa Cruz province, Argentina), inhabited by the parental plants of *Brachyphyllum garciarum* was suggested by Carrizo et al. (2019, this issue) based on the cuticle foliar features. This region was probably quite diverse as for other Argentinian material (San Juan Province), high temperatures and low humidity was suggested for *Zuberia papillata* (Martinez et al., this issue).

Theoretically, the most accurate comparisons occur within the same species from different palaeoenvironments, representing extremely rare fossil material; however the characteristics of the palaeoenvironment could be determined through two different studies. These studies could answer at least partly to the questions of Bajpai and Maheshwari (2000), wondering whether species differences (between *Pachypteris indica* and *Komlopteris indica*) were actually species or genus differences, as in their study only one species in each genus was reported.

The first example is the comparative studies made between specimens of the same species (*Suturovagina intermedia*), from two different localities, and referring to two different macro-paleoenvironments (= palaeoenvironments of the localities sensu lato), versus micro-palaeoenvironment which is the environment just around a plant or a leaf (e.g. differences of sun and wind and rain between upper part and lower part of leaf; differences between the top and the bottom of the plant, between the center and the outer part of a plant especially for plants with many branches like tress... One is more xerothermic (Mairot et al. 2014) and the other is less xerothermic (Yang et al. 2018), as evidenced by the associated floristic elements and the EDS data of the matrix. Although the cuticles are generally similar in microstructure, significant differences in ultrastructure (their Table 3) were observed between them. Those of the leaves from the less xerothermic locality (Yang et al; 2018, their Fig. 12), show not only a reduction of the thickness, but also different proportions of the layers (wavy A1, A2, B1, and B2) of ordinary epidermal and subsidiary cell cuticles. Based on statistics, the authors proposed 12 ultrastructural characters of ecological significance for this species. Although data are obtained from very limited observations, a potential palaeoenvironment significance of FCA axes (from Fig. 7; Appendix G) was proposed here (Fig. 9), taking account of the cuticle ultrastructure of *Suturovagina* from two localities. Among the 15 characters of these

analyses, the main characters responsible in construction of the FCA axes are A% and A2% of the cuticle proper, B% and B1% of the cuticular layer. An increase in thickness of the cuticular membrane also indicates a more xerothermic environment (South East China), and it is further related to the thickness proportion of A2 and A1 and also the thickness of B2 layers, whereas B1% decreased as compared with that of less xerothermic (North East China) cuticles (Fig. 9). Furthermore, although they are not important for the construction of the FCA axes, more xerothermic (South East China) palaeoenvironments are related to thicker opaque lamellae of the A1 layer and thinner translucent lamellae. This is a demonstration of the signature of environment, moreover it is also of interest for the probable role(s) and equilibrium played by each layer for the elaboration of the cuticular membrane. It would be very enlightening to obtain more material from the same species in other types of palaeoenvironments.

The second study is different as it compared two kinds of leaves of the same species of *Komlopteris nordenskioeldii* (Nathorst) Barbacka (Guignard et al. 2001) from one single locality. Compared with some living leaf plants related to this fossil taxon, fossil sun and shade leaves (different in their macro-morphologies) were selected, possibly referring to potentially high sensibility to micro-palaeoenvironmental influences within each sun and shade leaf two types (in macro- and microscopy). The sun leaf cuticles of ordinary epidermal cells (more exposed to wind, rain...) were thicker than those of the shade leaf cuticles (less exposed to wind, rain...). Moreover, the sun leaf upper cuticle was more exposed than the lower cuticle. Similar is the case with the shadow leaf. In both types of leaves, therefore, the upper cuticle was thicker than the lower cuticle. In total, there are four types of leaf cuticles with different thickness (sun upper and lower leaf cuticles, shadow upper and lower leaf

cuticles; Fig. 10). Under TEM, these cuticles were considerably more elaborate in their ultrastructure. Fibrils, pillar-shape structures made of condensed fibrils, granules, and amorphous material are the constituent parts (their Fig. 1). The proportions of the four types of material show distinct changes in the four types of cuticles, demonstrating the great sensibility of this taxon to micro-palaeoenvironment differences. From shaded lower cuticles (less exposed) to sun upper cuticles (most exposed), a gradual decrease of fibrils (and pillar-shape structure), and gradual increase of amorphous material can be observed. The intermediate cuticles (sun lower and shade upper cuticles) contain more granules, which are fewer or absent in the extreme cuticles (sun upper and shade lower cuticles).

Finally, ultrastructural studies of cuticles of living plants (Coniferales, genus *Pinus*, Bartiromo et al. 2012; Angiosperms, genus *Erica*, Bartiromo et al. 2013), also from the same species living in two different environments (volcanic/non-volcanic areas) could be significant for comparisons with fossil materials, as the precise data of these environments can be analysed (soil and atmospheric CO₂ concentrations, wind direction...). In *Pinus halepensis*, the cuticular membrane CM thickness with different zones of B1 increased in the volcanic area, and the different zones of B1 also varied. However, the cell wall (CW) could be measured for this living material (it is rarely the case with fossil material as it is more or less damaged or absent in most cases). The CM was balanced by a thinner CW, making the whole thickness of CM + CW significantly equal between the two environments. In *Erica arborea* from the same localities, significant differences of CM + CW were found between the two environments, also between the two CW and the two A2 layers. With these two types of living plants, it is clear that the CW also participates in the isolation of the plant. As the CW is rarely observed or entirely preserved in fossils, most often the rare parts with CW do not allow for statistical measurements, so extant cuticle studies provide a great advance in the

knowledge of cuticles. In studying cuticle ultrastructure of these living plants, Bartiromo et al. (2012, 2013) mentioned that ‘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’. Such relation between granules-fibrils and the environment was cited and discussed in the paper of fossil *Ginkgoites skottsbergii* (Guignard et al. 2016) (having a thicker granular A2 layer and thinner B1 fibrilous layer), which shows a different equilibrium of the layers, compared with the more xerothermic *G. ticoensis* (having a thinner A2 granular layer and a thicker B1 fibrilous layer).

3.3 Evolution implications of fossil plant cuticles

Plant cuticle evolution is more and more enhanced for various aspects (Niklas et al. 2017, Petit et al. 2017, Renault et al. 2017 and 2018; especially in tomato, Yeats et al. 2012 and Yeats and Rose 2013), and as written by Cohen et al. (2017) “Certainly, the development of plant lipophilic barriers had an immense impact on life on earth and thereby is often considered as one of the most significant evolutionary events” (Kenrick and Crane, 1997; Bateman et al., 1998). However, fossil remains are still scarcely taken in account. When working on plant cuticle ultrastructural features, evolution has been a very attractive topic, enhanced by authors studying fossil plants, from the beginning of TEM papers in fossil cuticles: in their last paragraph of their *Tarphyderma* study, Archangelsky and Taylor (1986) suggested that ‘various structural features were evolving in other major groups of plants in response to the habitat’. The understanding of evolution was also enhanced through the analysis of chemical compounds (Taylor 1999), for the ‘colonization of the terrestrial environment’ (Edwards et al. 1982), and conservation of ‘biosynthetic pathways’ for the

‘evolutionary radiation’ of ‘basal land plants’ (Jeffree, 2006), and more particularly related to a thickened cuticle in Cactaceae evolution (Ogburn and Edwards 2009). This topic is potentially promising as in living plants recent molecular biology studies in very various taxa enhanced more and more genes related with cuticles, in agronomic plants (fleshy fruits and cereals; Taketa et al. 2008; Alkio et al. 2012; Lashbrooke, et al. 2015; D’Angeli et al. 2016; Wang et al. 2016; Bi et al. 2017; Xu et al. 2018; Zhang et al. 2018; Shi et al. 2018; Belge et al. 2019; Cohen et al. 2019; Fasoli et al. 2019; Wang et al. 2019), and model plants (Xu et al. 2019, Zhao et al. 2019). Until recently, although cuticles have been known from very old periods and in various taxa [from terrestrial algae (Good and Chapman 1978) to angiosperms], and suggested to have evolutionary significance (Edwards et al. 1982, Riederer 2006, Niklas et al. 2017), there are still limited evolutionary data available in fossil plants using fine cuticle details, mainly owing to limited taxa studied; however, some points can be developed.

Through all orders, considering all the data available for comparisons in the cuticle formulae (Figs. 2 and 3), from the most simple formula to the most complex one, a potential evolution of formula through the orders is not clear at all. For instance, Cycadales (basal Gymnosperms compared with Coniferales) have a simple formula, Ginkgoales (also basal Gymnosperms) have a quite complex formula, while Coniferales have different types of formulae.

However, there are some interesting clues recently revealed by cuticle ultrastructure studies of fossil cuticles as, at the level of the order, Coniferales can be interestingly evocated. Extant Coniferales are studied more and more (Leslie et al. 2015; Rothwell et al. 2018) in phylogeny through leaf anatomical characters and chloroplast genes (Taxaceae, Elpe et al. 2018; Pinaceae including fossils, Gernandt et al. 2016 and 2018a and b, Saladin et al. 2017;

964 Cupressaceae and other families including fossils, Leslie et al. 2018), but none of these
965 approaches used fine details of the cuticle ultrastructure. Although fossils Coniferales are up
966 to now only represented by four Families in TEM (Araucariaceae, Cheirolepidiaceae,
967 Miroviaceae, Podocarpaceae; Figs. 2 and 3; appendix C), some remarks can be done.
968 Araucariaceae have the simplest formula (A2 and spongy B1) and they emerged in Palaeozoic
969 (Taylor et al. 2009), Triassic (Kunzmann 2007; Stockey 1982 and 1994) putatively in Late
970 Triassic (Axsmith and Ash 2006) while unambiguously in Early Jurassic (Leslie et al. 2018).
971 Cheirolepidiaceae have a more complex cuticle (wavy A1, A2, B1, B2) and they emerged
972 widely speaking later (in Mesozoic, Taylor et al. 2009), more precisely in Late Triassic to Late
973 Cretaceous (Yang et al. 2018). Miroviaceae are the most complex in their cuticle formula
974 (with A1U (+ A1L for some cuticles), A2U (+ A2L for some cuticles), B1 for *Mirovia*
975 *sibirica*) and this species is the youngest, dated to the Early Cretaceous. It seems that,
976 comparing the cuticles at the same age (Early Cretaceous, the age of the localities studied for
977 these three Families), although we have to be very cautious a potential relation between the
978 degree of simplification and emergence period exists, the oldest family having the most
979 simple cuticle and the youngest family having the most complex cuticle. The three-
980 dimensional FCA (Fig. 11), with the combination of 3 axes, made with the absolute values
981 and % of characters (also their absence as evaluated with values “0”), shows clearly with
982 almost 83% data characteristic that the three families are in accord with their emergence
983 period (Fig. 11b; the oldest is on one side of the 3D scheme, the youngest on the opposite
984 side), while Cheirolepidaceae and Miroviaceae are partly merged for some cuticles because of
985 similar values and % of layers along the three axes of the FCA. Although the number of
986 cuticles studied for their fine ultrastructural details is different in each family, they show great
987 differences in their variability of cuticle characters (means and % of characters; appendix B of
988 data in brief article). Araucariaceae are the most stable in their characters values, moreover

989 this stability is reinforced by the other Araucariaceae studied in TEM [Del Fueyo 1991, Del
990 Fueyo and Archangelsky 2002) are from the same age (Lower Cretaceous)], which show the
991 same cuticle formula. Cheirolepidiaceae cuticles show some variations in their cuticular
992 ultrastructural characters values, they are from the same age but quite related with
993 palaeoenvironment at least for some of them. Miroviaceae cuticles show the greatest
994 variations in their character values, they are from different ages and show different cuticle
995 formulae enabling some remarks detailed further for the potential evolution among this
996 family.

997
998 In constructing the preliminary tentative evolutionary scheme of the cuticle ultrastructure in
999 Coniferales, 8 characters (among a total of 23) are much more informative than the others
1000 (Fig. 11a). Firstly, spongy B1 layer of the cuticular layer (its mean and %, numbered 18-19 in
1001 the figure) is the main distinguishing character among taxa, making mainly the F1 Axis (the
1002 most important in significance, 39.9%). Secondly, A1L (its mean, numbered 8) and A2L (its
1003 mean and %, numbered 14-15) of the cuticle proper, making mainly the Axis 2 (second in
1004 importance, 26%) are also of evolutionary significance. Finally, A2U (its mean, numbered 12)
1005 and B2 (its mean and %, named 22-23) of the cuticle proper make mainly the Axis 3 (the third
1006 in importance, 16.6 %).

1007
1008 In this study, the 15 remaining characters (for instance the total cuticular membrane thickness)
1009 are located around the axes and not so important to the evolution scheme tentative of cuticle
1010 ultrastructure. Till now very few studies have been carried out on the evolution of the cuticle
1011 ultrastructure. The present study on the Coniferales is the first attempt to response to Taylor's
1012 (1999) early assertion, writing that "...the cuticle ultrastructure in palaeobotany may also
1013 provide an indirect method of determining how cuticles have evolved.". Similar evolutionary

trend in cuticle structure was observed in another Order, Umkomasiales (Labe and Barale, 1996), where the authors noticed an increase in the complexity of cuticle ultrastructure of 4 taxa from Early to Late Jurassic, but without statistical data.

At the level of the family, the cuticle ultrastructure is variable among genera and species. However, potential evolutionary trends are also visible. In Miroviaceae (Nosova et al. 2016), three species of the genus *Mirovia* from different ages were studied by using TEM: *M. kazachstanica* from Early Jurassic (Toarcian), *M. macrophylla* from Middle Jurassic (Bajocian) and *M. sibirica* from Early Cretaceous (Berriasian-Valanginian). Although it is not at all the remarks of these authors, as for the three Families of Coniferales just evocated, the complexity of the cuticle ultrastructure of these three Miroviaceae species is increasing through time: A1, A2 and B layers visible in the oldest species; A1, A2U and A2L, B in the younger Middle Jurassic species; A1U and A1L, A2U and A2L, B in the youngest species.

Secondly, another very different Family case concerns Ginkgos, where macromorphological (gross morphology...) characters were studied in phylogeny (Hasebe 1997; De Franceschi and Vozenin-Serra 2000), more precisely for TEM ultrastructure of reproductive organs (Rohr 1997). In 2003, allied with some studies (Zheng and Zhou 2004) comparing reproductive characters (ovules) and vegetative characters (leaves) between several ginkgos from different ages (from 170 million years ago to living *Ginkgo biloba*), Zhou and Zheng related a possible case of peramorphosis, which is a case of heterochrony. Using the same material than Zhou and Zheng (2003), cuticle ultrastructural details (the different layers, their values and percentages), allied with macromorphological characters of ginkgos, give some fruitful details within *Ginkgo* (Guignard and Zhou 2005). As result of comparisons between the oldest

Ginkgo in China (*G. yimaensis*, 170 millions of years) and living *G. biloba*, male and female), if the cuticle ultrastructure of these leaves show the same layers of cuticles (A1U + A1L, A2, B1) allowing a characteristic of this genus, between the two taxa separated by 170 millions of years, only few differences in the thicknesses and % of each layer were revealed by statistical measurements, showing a high stability and though a very small degree of potential Evolution compared with the changes of macromorphology of the leaves in the same period. It is interesting to note that, for the same type of organ, i.e. leaves, macromorphological characters (as surfaces and lobes of the leaves) did not follow the same scheme than some micromorphological characters (cuticle ultrastructural characters). Further works are, therefore, needed to study the cuticles of other fossil species of Ginkgos from the Cretaceous and younger strata, such as *G. apodes*, *G. jiayinensis*, *G. adiantoides* and *G. cranei* (Zhou and Zheng 2003; Zhou et al., 2012; Zhou, 2009; Quan et al., 2010; Quan and Zhou, 2010), to check if there are any changes in ultrastructure of the layers.

Last point concerns the “adaptation” topics, frequently used in fossil cuticle fine detail studies and a component of evolution. Generally speaking, cuticles are known to be related with the arrival of plants on lands: in the introduction of the book on biology of the plant cuticle, Riederer (2006) enhanced in the “evolution of the plant cuticle” part “... the simultaneous appearance of cuticles and stomata as evidence for the physiological adaptations to the colonization of the land...”. In extant plants (Mesembryanthemaceae), Ihlenfeldt and Hartmann (1982) considered “fine structure of the epidermal wall” (actually including cuticle according to their figures) as one of the characters to “describe variation in the xeromorphic type of epidermis”. In fossil Bennettitales TEM cuticle study (Barale and Baldoni 1993), “adaptation” in a very wide sense is cited.

1066

1067 According to Taylor et al. (2009) in their fossil plants book's glossary, adaptation is "a
1068 structure or feature that performs a particular function and which reveals in increased survival
1069 or reproduction". In extant organisms the definition is quite similar, enhancing the non-
1070 reversibility of adapted features and the transmission to next generations. Adaptation is
1071 usually opposed to accommodation, which is also a change but related with the phenotypic
1072 plasticity, reversible and not transmitted to the next generations. It is clear that in fossil plants
1073 studies these two aspects (adaptation – accommodation) cannot be distinguished as conversely
1074 to extant plants neither changes of environment for one single plant nor reversibility and
1075 transmission to the next generations can be experimented. For instance in living plants where
1076 two different environments for one taxon were studied among two species (Bartiromo et al.
1077 2012, 2013), although the authors observed significant differences between the cuticles of
1078 volcanic and non-volcanic environments, very cautiously they did not use at all "adaptation",
1079 as neither non-reversibility of the changes nor transmission to the next generations was
1080 experimented for an eventual adaptation and/or accommodation case.

1081

1082 In TEM studies of fossil cuticles "adaptation" or "adapted" is actually used particularly in a
1083 very wide sense of unusual features, especially for extreme palaeoenvironments like
1084 xeromorphic or volcanic ones, where unusual cuticle fine details are observed. Haworth and
1085 McElwain (2008, 2009) concentrated on the critics of "ecological functions of "xeromorphic"
1086 adaptations..." including of course cuticles but unfortunately no ultrastructural fine details.
1087 Villar de Seoane (1999), in a Bennettitales TEM study, enhanced "...strategies of adaptation
1088 to the extreme environment..." (volcanic), not especially for the cuticle details but for the
1089 stomatal apparatus characters. Interestingly, ultrastructural cuticle fine details are included in
1090 the remark of Thevenard et al. (2005) about a Pteridospermales taxon (*Dichopteris*), for non-

xeromorphic characters considering this cuticle with no unusual details. It is clear that several “evolutionary” terms are used in the papers, more or less mixed and apparently in various meanings, also very linked with palaeoenvironmental considerations. With the increase of data, these evolutionary aspects seem to become clearer, however as a synthesis of the use of these terms seems irrelevant, following papers are cited just to illustrate these remarks.

Particularly for volcanic palaeoenvironment, in Cycadales *Ticoa* study Archangelsky et al. (1986) enhanced for the cuticle ultrastructure to consider “...evolutionary significance of these features”. In Cycadales too, *Pseudoctenis* TEM cuticle details (Archangelsky et al. 1995) is related potentially with the stress in volcanic areas. In Cycadales (Villar de Seoane 2005), xeromorphy is used for volcanic palaeoenvironment. In Coniferales, *Tarphyderma* study (Archangelsky and Taylor, 1986) showed a very special stomatal apparatus and “adaptations” and “structural adaptations in fossil plants” are mentioned. In Bennettitales (Villar de Seoane 2001), «...xeromorphic characters...» are used to discuss in seven species study including other features than TEM details. Carrizo et al. (2014) wrote about the “great success at adapting to the different stressed environments” for a new *Ruflorinia* (Pteridospermales), through various features including TEM. Recently (Passalia et al. 2010) used the term “clade” for the study of a new Cycadales, and suggest a “Chigua clade” in “plant evolution”.

In Cheirolepidiaceae where special characters are usually observed (wavy A1 layer and other fine details...), xeromorphic features and adaptations are largely cited. Villar de Seoane (1998), comparing extant taxodiacean and fossil cheirolepidiacean taxa, asking in the discussion for the “interpretations of the phylogenetic relationships of the Cheirolepidiaceae with other conifer families...”, suggested in the “relationship” conclusion about “a close

relationship between leaves of the Cheirolepidiaceae and Taxodiaceae”. *Suturovagina* and *Pseudofrenelopsis* studies (Yang et al. 2009, Mairot et al. 2014, Guignard et al. 2017, Yang et al. 2018) also cite these words.

4 Conclusions and perspectives

Besides a brief review of the research history and methods of the plant cuticle, the achievements made in recent 33 years, of cuticle ultrastructure in the study of fossil plants, are summarized below. Since then, a number of contributions were made to explore the importance of cuticle ultrastructure in studying the taxonomy, palaeoenvironment and evolution of fossil plant cuticles (mainly leaf cuticles). TEM cuticle researches are most commonly applied in taxonomic interpretation of fossil plants. Including in the diagnosis and description of taxa, characteristic cuticle ultrastructure features are useful in comparison between different taxa. As it shows in the analyses of the 52 selected cuticles of fossil plant, simple formulae of ultrastructure characters showing the qualitative differences, i.e. the presence or absence of different layers and sublayers, could be efficiently applied in taxonomy at the level of order and/or family. More complex formulae showing the quantitative differences among 41 selected cuticles (i.e. measurements and statistic methods and two- and three-dimensional multifactorial analyses; HAC, FCA, CHIAS) could be efficiently applied in taxonomy at lower taxonomic levels. It should be noted that the taxonomic importance of ultrastructure characters may be different among orders and families or within one family, such as the case in the Cheirolepidiaceae. In recent years, palaeoenvironmental interpretation has become the second topic for the cuticle ultrastructure studies of taxa and even the same taxon from different localities. In some taxonomical groups,

e.g. Cheirolepidiaceae where cuticle ultrastructural data are rather numerous, the signature of xeromorphy appears to emerge among taxa at various degrees. It depends obviously on the nature of the environments (micro- or macro-palaeoenvironment), and also the influences on different components of the cuticles (or even for instance cell walls, not belonging to cuticle but of course directly connected to it) are not impartial. In analyses of the cuticle ultrastructure data of selected taxa, it is of interest to note that some evolutionary trends have been detected among fossil coniferous group of different geological ages. The oldest family Araucariaceae yield the simplest ultrastructure, and the younger the more complex. In the youngest family Miroviaceae, the cuticle ultrastructures of the three species of the genus *Mirovia*, ranging from Early Jurassic to Early Cretaceous, show progressive complexity. In Ginkgoaceae, however, the contrary is the case: cuticle ultrastructure of the extant species of *Ginkgo* shows little difference as compared with that of the Jurassic species. As material suitable for cuticle ultrastructural study with the available methods is limited, exclusive of many other plant groups than gymnosperms with thick cuticles, it is still a very young and developing field in palaeobotany and till now accumulated data, especially those of statistic analyses, are meager. Nevertheless, the progress so far made in this field is interesting, as it opens a new window allowing an insight into the subtleties of cuticle ultrastructure, and their bearing on the taxonomy, palaeoenvironment and evolution of the fossil plant fine details in the geological ages. Further studies and more sophisticated methods in this field are urgently awaited.

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Caption of plate, tables, figures, appendices

Plate I. Illustrations of the Transmission electron microscopy different types of layers and sublayers from Figure 1. OEC = ordinary epidermal cell cuticle, GC = guard cell cuticle.

1-2. A1 straight polylamellate layer. Note the A2 granular layer below ,and extracuticular material over the A1 layer. 1. rare case in *Ginkgoites ticoensis*, OEC. 2. rare case in *Pseudofrenelopsis gansuensis*, OEC. 3-5. A1 wavy polylamellate layer. Note the A2 granular layer below, and extracuticular material over the A1 layer. 3. *Pseudofrenelopsis gansuensis*, OEC. 4. *Suturovagina intermedia*, OEC. 5. *Pseudofrenelopsis gansuensis*, OEC. 6-7. A1U and A1L polylamellate sublayers. Note that the A1 layer is divided in two parts, A1U (U = upper) sublayer (or zone) with straight polylamellae and A1L (L = lower) sublayer (or zone), with fewer lamellae oriented more or less oblique or even rather transversal to the outermost part of the cuticle. 6. *Ginkgoites ticoensis*, OEC. 7. *Ginkgoites ticoensis*, OEC. 8-9. A2 granular layer, homogeneous or heterogeneous. Note the granules of this layer, very homogeneous for A2U (U = upper) or much more heterogeneous for A2L (L = lower). 8. *Pseudofrenelopsis gansuensis*, OEC. 9. *Mirovia sibirica*, OEC, Courtesy Nosova. 10-11. B1 layer, fibrilous or spongy. Note that the fibrils are more or less mixed with delicate granules, they can make a fibrilous scheme or spongy if the concentrations of fibrils is variable and then more or less visible. 10. *Brachyphyllum garciarum*, GC. 11. *Ginkgoites ticoensis*, OEC. 12-14. B2 granular layer. Note the granules of this thin layer in the inner most part of cuticles, directly connected with B1 fibrilous layer just above, above blackish cell remnants as in photo 13. 12-14. *Suturovagina intermedia*, OEC. For other photos of these cuticle taxa, see Del Fueyo et al. 2013 (for *Ginkgoites ticoensis*), Guignard et al. 2017 (*Pseudofrenelopsis gansuensis*), Nosova et al. 2016 (*Mirovia sibirica*), Mairot et al. 2014 and Yang et al. 2018 (*Suturovagina intermedia*), Carrizo et al. 2019 this issue (*Brachyphyllum garciarum*).

Table 1 selected criteria for various methods

1988 Note. Percentages and duration values are indicated when they are provided in the relevant
1989 papers.

1990

1991

1992

1993 Figure 1. Synthetic construction of a fossil cuticle.

1994

1995 Note. This scheme is based on Archangelsky et al. (1986) following Holloway (1982), and
1996 taking account of new data from Guignard and Zhou (2005) and Nosova et al. (2016).

1997

1998 Figure 2. Potential ultrastructural dichotomous key for fossil cuticles.

1999 To appear in colour

2000

2001 Note. a. cuticles with A1, A2 /or not, B1 /or not, B2 /or not

2002 b. cuticles with no A1, A2, B1 /or not, B2 /or not

2003

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2005 Figure 3. HCA made with cuticles formulae

2006 To appear in colour

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2010 Figure 4. CHAID made with guard cell cuticles features.

2011 To appear in colour

2012

Note. The guard cell cuticle can be made with A1L (upper zone) and A1L (lower zone), A2, B1 and B2 layers and sub-layers. The present CHAID enables to distinguish each type of the 11 guard cell cuticles according the presence-absence of firstly A1U, then A1L or A2, then B1 or B2.

Figure 5. FCA three-dimensional representation of 23 cuticle characters, linked with figure 6. To appear in colour

Note. Two visualisations of the 3D representation are given, showing the relations between characters according 2 orientations of the three axes of the FCA. Because of their rarity among the cuticles, 6 characters are supplementary data: A2L (mean and %), B1 spongy layer (mean and %), B2 (mean and %). Data are from appendix B of data in brief article.

Figure 6. FCA three-dimensional representation of 41 cuticles, linked with Figure 5.

To appear in colour

Note. Two visualisations of the 3D representation are given, showing the relations between cuticles according 2 orientations of the three axes of the FCA. UC and LC = upper and lower cuticles. Coniferales are represented in purple colour by : Cheirolepidiaceae (*Suturovagina intermedia* more xerothermic LC, less xerothermic thick cuticle LC, less xerothermic thin cuticle LC; *Pseudofrenelopsis dalatzensis* LC; *Pseudofrenelopsis gansuensis* LC);

Miroviaceae (*Mirovia kazachtanica* UC and LC; *Mirovia macrophylla* UC and LC; *Mirovia sibirica* UC and LC); Araucariaceae affinity (*Brachyphyllum garciarum* UC and LC). Ginkgoales type I are represented in light green colour by: *Sphenobaiera huangii* UC and LC; *Baiera furcata* UC and LC. Ginkgoales type II are represented in dark green colour by: *Ginkgo biloba* male UC and LC, female UC and LC; *Ginkgo yimaensis* UC and LC; *Ginkgoites ticoensis* LC; *Ginkgoites skottsbergii* LC; *Pseudotorellia asiatica* UC and LC; *Pseudotorellia samylinae* UC and LC. Pteridospermales type I are represented in red colour by *Komlopteris nordenskioeldii* UC and LC sun leaf, *Komlopteris nordenskioeldii* UC and LC shade leaf; *Dichopteris visianica* UC and LC. Pteridospermales type II are represented in red colour by; *Pachypteris gradinarui* UC and LC. Czekanowskiales are represented in dark blue colour by: *Arctobaiera renbaoi* UC and LC; *Phoenicopsis eutyphylla* UC and LC. Data are from appendix B of data in brief article

Figure 7. Two-dimensional FCA with 15 characters (a) and 9 cuticles from 4 taxa (b), belonging to 2 types: epidermal and subsidiary cells. Axes 1 and 2.

To appear in colour

Note. mean = mean thickness in μm ; CM = total cuticular membrane thickness mean; Ol and TL = opaque and translucent lamellae thickness. OEC, SC, GC = ordinary epidermal, subsidiary, guard cell cuticles. *P. gansuensis* = *Pseudofrenelopsis gansuensis*; *P. dalatzensis* = *Pseudofrenelopsis dalatzensis*. *S. intermedia* = *Suturovagina intermedia*. more xe. = more xerothermic, less xe. = less xerothermic, thick c. = thick cuticle, thin c. = thin cuticle

2063

2064 Figure 8. 2 HCA with 9 (a) and 12 (b) cuticles from 4 taxa, 15 characters.

2065 To appear in colour

2066

2067 Note. OEC = ordinary epidermal cell cuticle; SC = subsidiary cell cuticle, GC = guard cell
2068 cuticle. *P. gansuensis* = *Pseudofrenelopsis gansuensis*; *P. dalatzensis* = *Pseudofrenelopsis*
2069 *dalatzensis*. *S. intermedia* = *Suturovagina intermedia*. more xe. = more xerothermic, less xe. =
2070 less xerothermic, thick c. = thick cuticle, thin c. = thin cuticle

2071

2072

2073 Figure 9. Potential synthetic palaeoenvironmental scheme for *Suturovagina* cuticles of
2074 Cheirolepidiaceae in China.

2075 Note. Data are from Yang et al. 2018, especially their Fig. 12. The cuticle membrane CM is
2076 made of A1 and A2 layers of the cuticle proper, and B1 and B2 layers of the cuticular layer.
2077 Although of less statistical interest, opaque lamellae OL and translucent lamellae TL of the A1
2078 layer are also represented in these two schemes.

2079 To appear in colour

2080

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2082

2083 Figure 10. Diagram of reconstruction of the four types of cuticles of *Komlopteris* according to
2084 their ultrastructures.

2085 To appear in colour

2086

2087 Note. The data from Guignard et al. 2001 were used.

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2091 Figure 11. Three-dimensional FCA, potential evolution scheme among Coniferales cuticles.

2092 To appear in colour

2093

2094 Note. a. Representation of the 23 characters. b. 13 cuticles of three Coniferales families.

2095 UC =lower cuticle, LC = lower cuticle. *Brachyphyllum* 1 and 2 = *B. garciarum* UC and LC,

2096 Araucariaceae affinity. *Suturovagina* 1 = *S. intermedia* more xerothermic LC. *Suturovagina* 2

2097 and 3 = *S. intermedia* less xerothermic thick cuticle LC and thin cuticle LC.

2098 *Pseudofrenelopsis* 1 = *P. dalatzensis* LC. *Pseudofrenelopsis* 2 = *P. gansuensis* LC. *Mirovia*

2099 1and 2 = *M. kazachstanica* UC and LC. *Mirovia* 3 and 4 = *M. macrophylla* UC and LC.

2100 *Mirovia* 5 and 6 = *M. sibirica* UC and LC. For original values and % data of each cuticle, see

2101 appendix C.

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2105 Appendix A. schemes of epidermis

2106 To appear in colour

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2108 Appendix B. original data for CHAID guard cell cuticles

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2111 Appendix C. curve tendencies for 41 cuticles

2112 To appear in colour

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2115	Appendix D. Cheirolepidiaceae original values and % for 12 cuticles
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2117	Appendix E. curve tendencies for 12 Cheirolepidiaceae cuticles
2118	To appear in colour
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2120	Appendix F. Cheirolepidiaceae, other axes of FCA with 9 cuticles from 2 types (axes 1-3, 2-3)
2121	To appear in colour
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2124	Appendix G. Cheirolepidiaceae, FCA with 12 cuticles from 3 types (axes 1-2, 1-3, 2-3)
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2126	Appendices of MethodsX article
2127	Appendix A. embedding technique of plant cuticles, for transmission electron microscopy
2128	To appear in colour
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2131	Appendix B. some steps of the sectioning, staining and observation of the plant cuticles, for
2132	transmission electron microscopy
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2138 Appendix A. 0-1 data for 52 cuticle formulae

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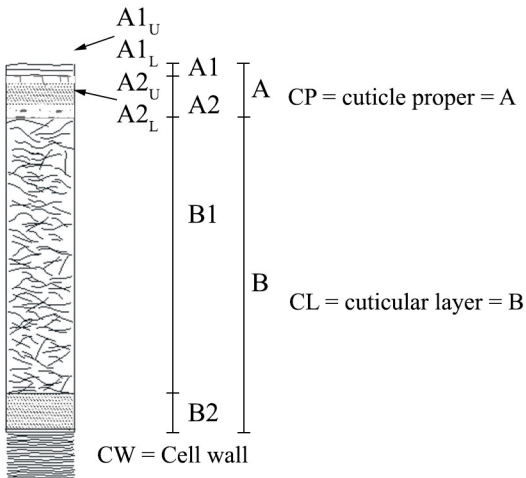
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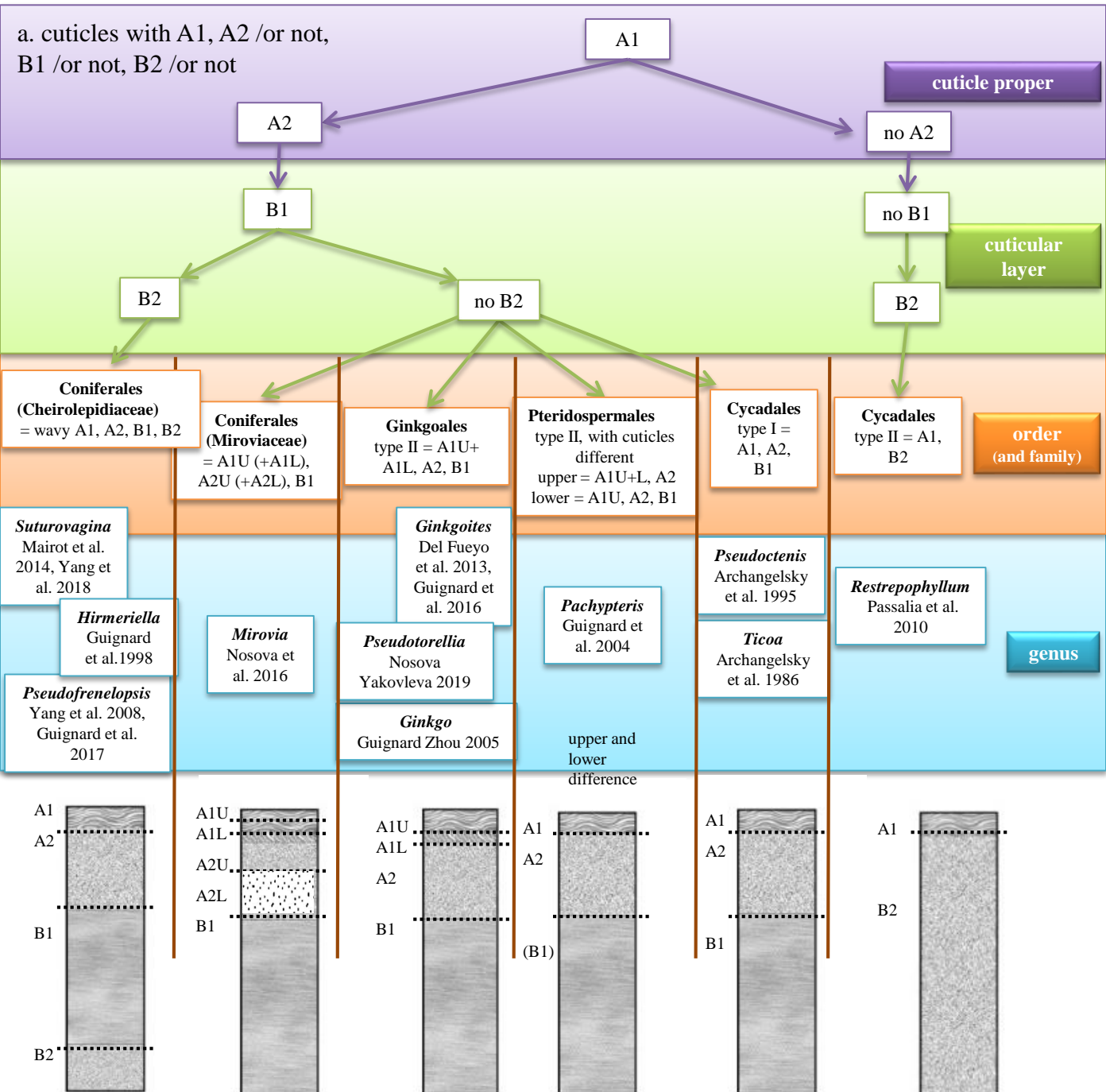
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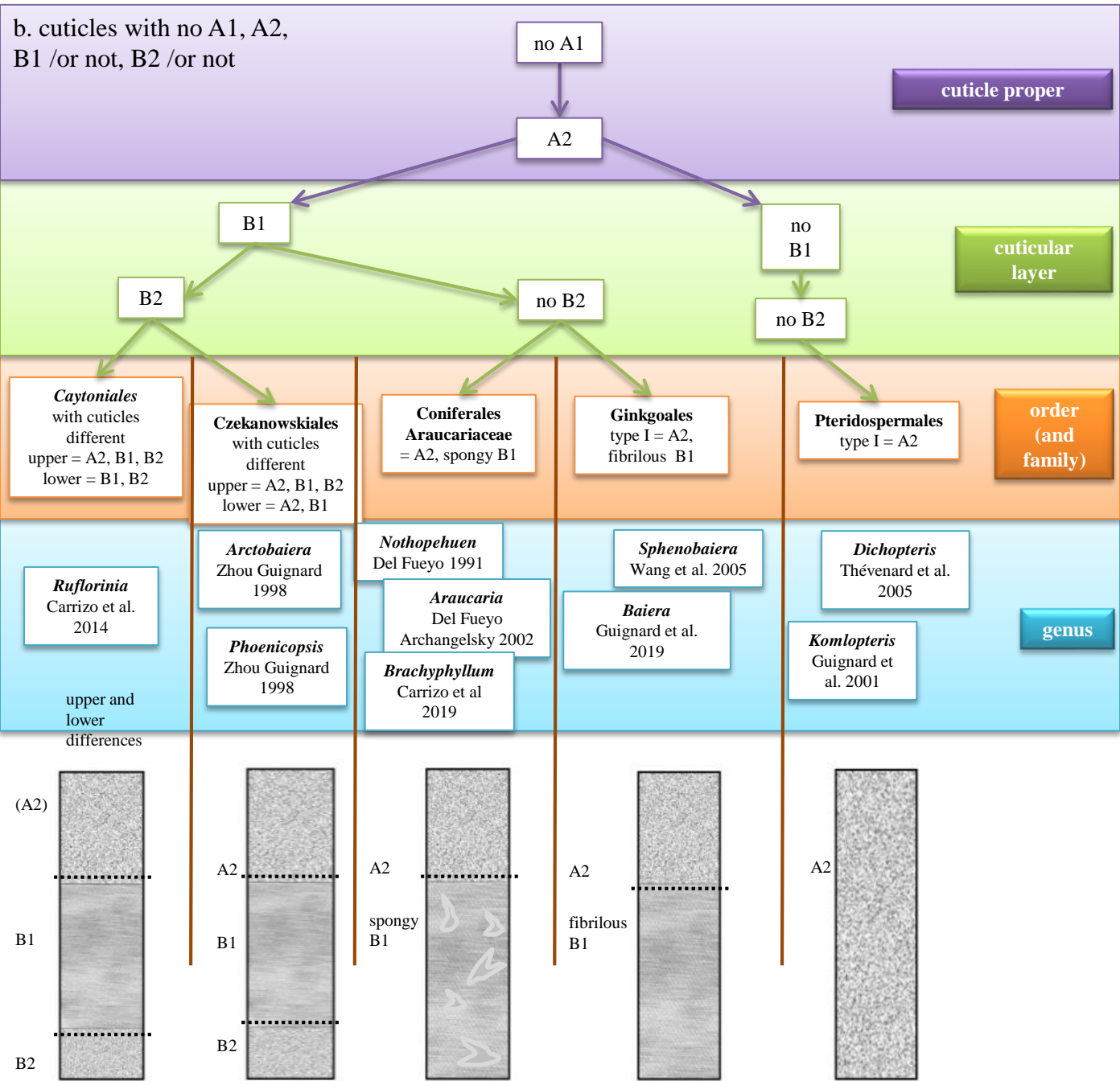
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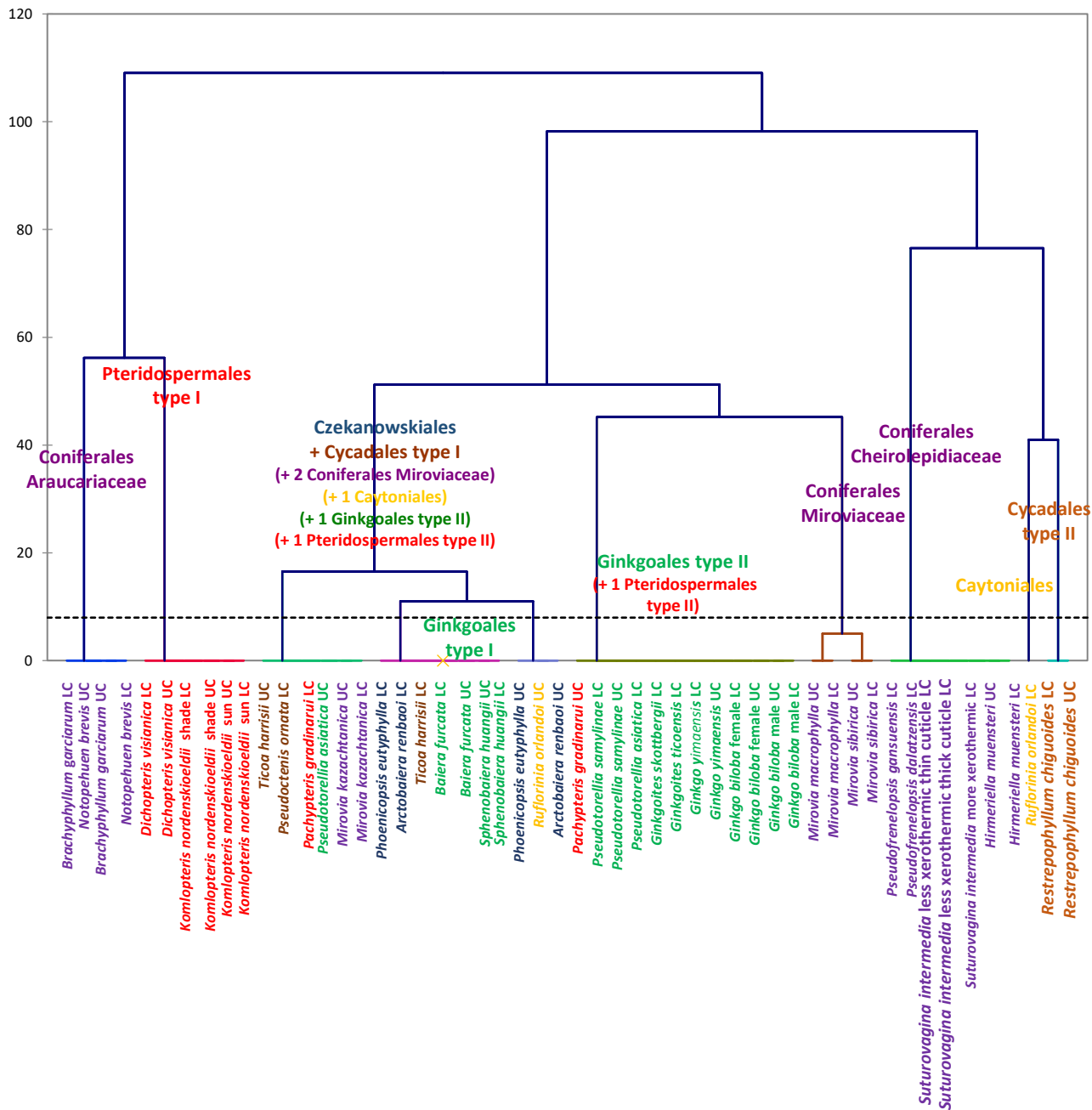
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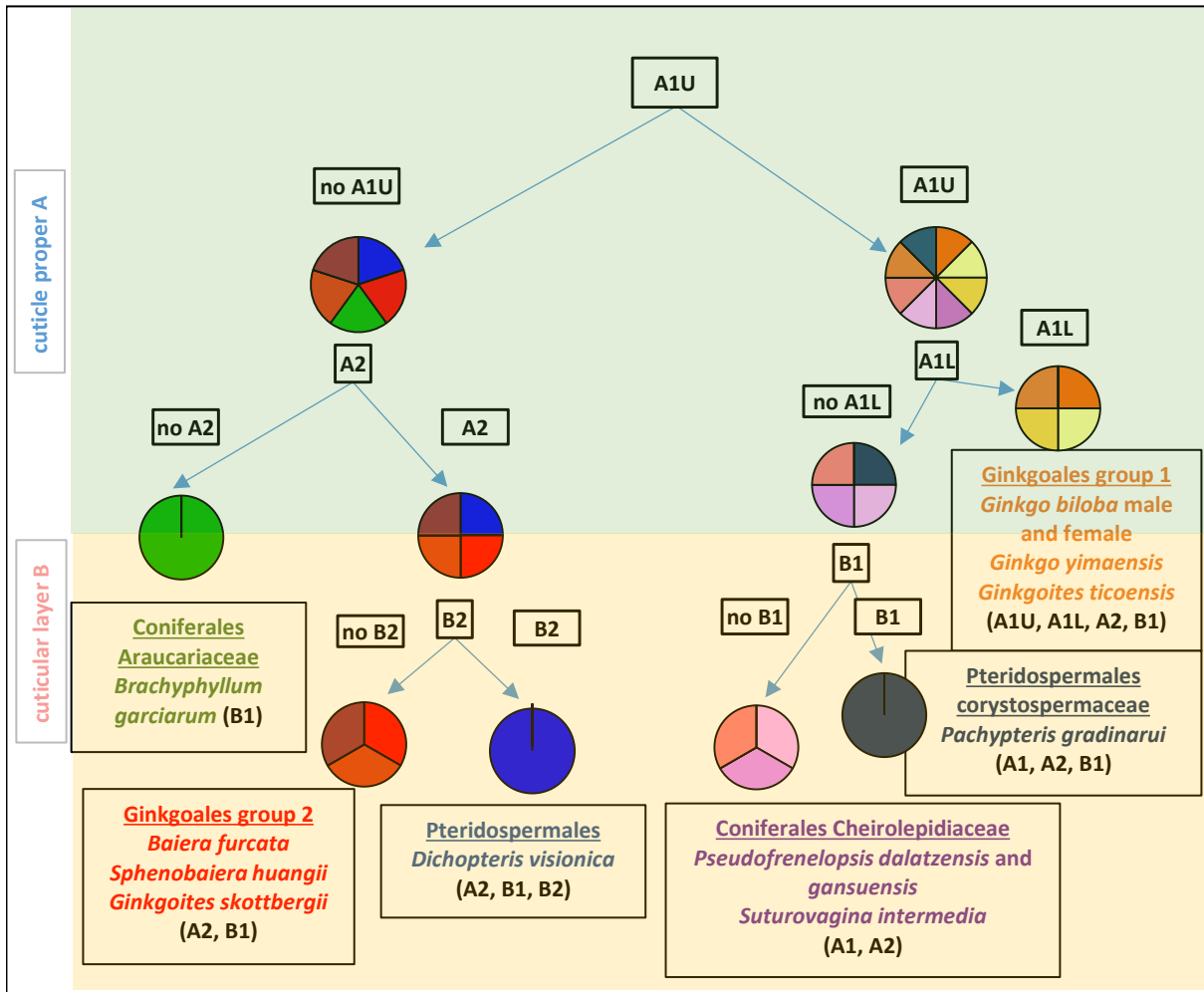


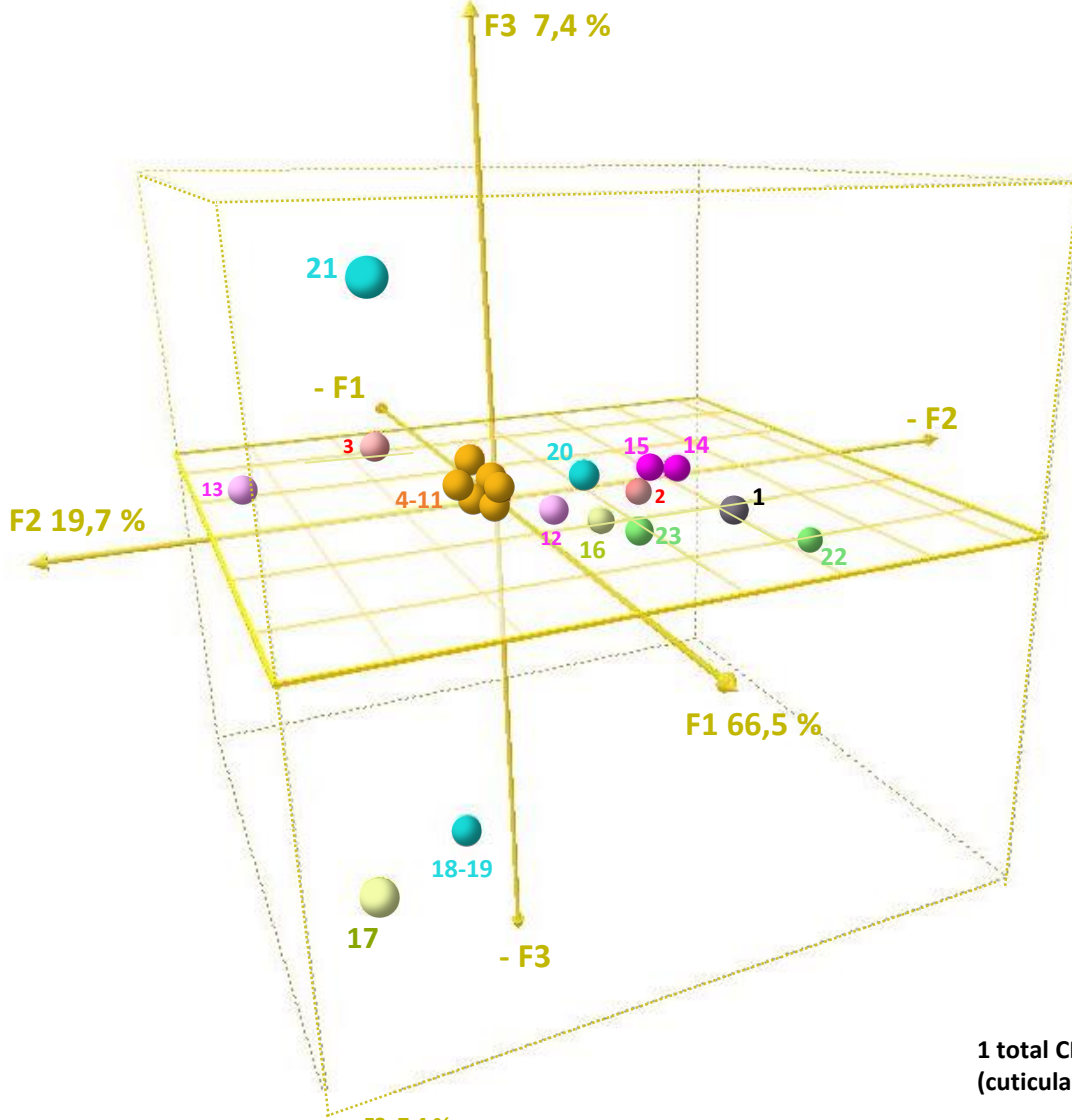


b. cuticles with no A1, A2,
B1 /or not, B2 /or not









**1 total CM mean
(cuticular membrane)**

cuticle proper A

2, 3 A mean and A %

4, 5 wavy A1U mean and %

6, 7 straight A1U mean and %

8, 9 A1L mean and %

10 OL opaque lamellae

of A1 mean in nm

11 TL translucent lamellae

of A1 mean in nm

12, 13 A2U mean and %

14, 15 A2L mean and %

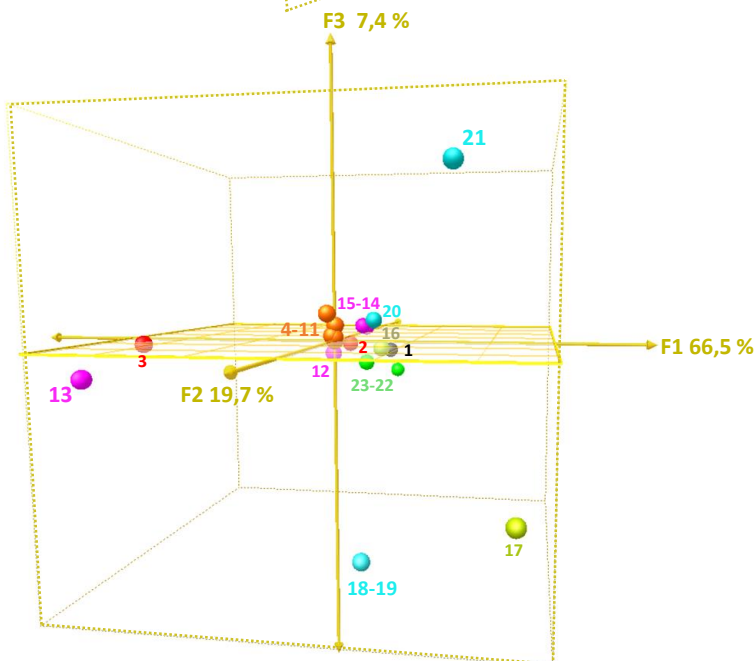
cuticular layer B

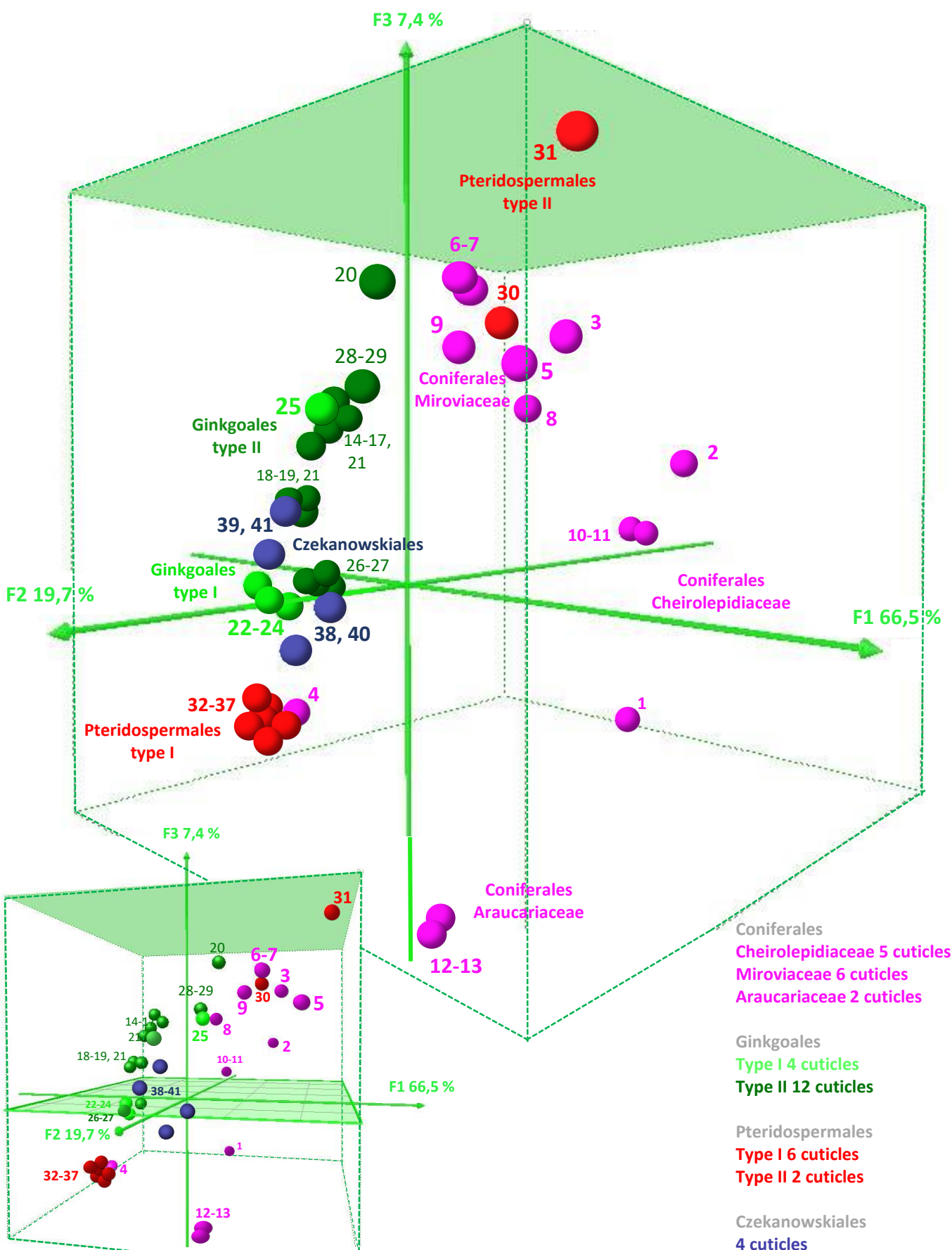
16, 17 B mean and %

18, 19 spongy B1 mean and %

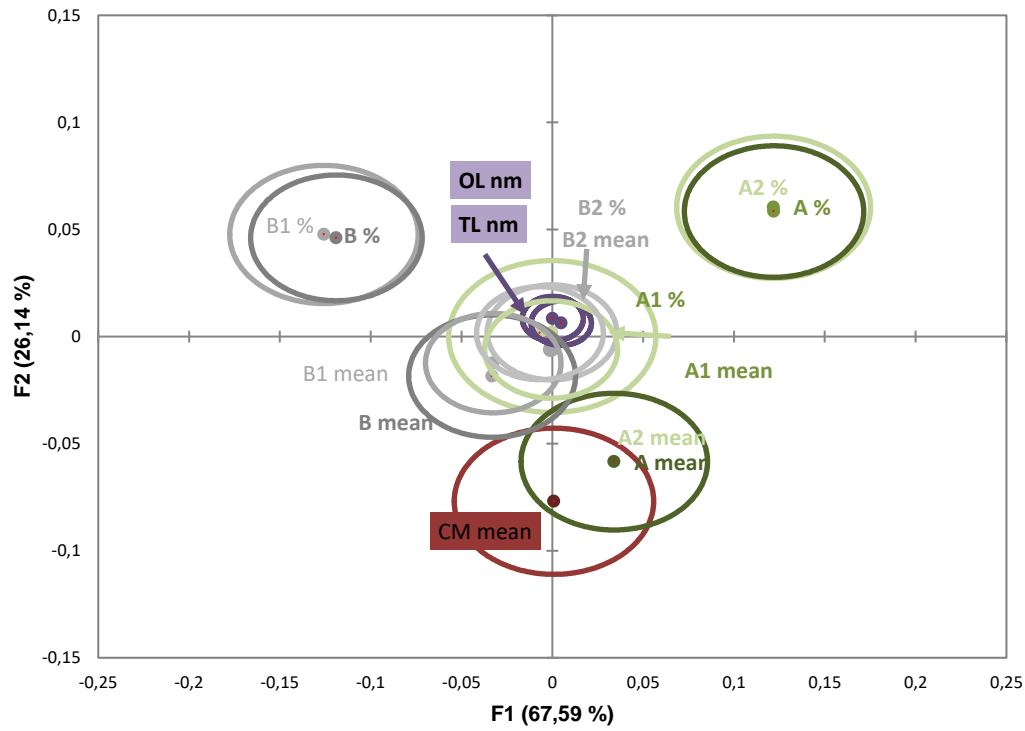
20, 21 fibrilous B1 mean and %

22, 23 B2 mean and %

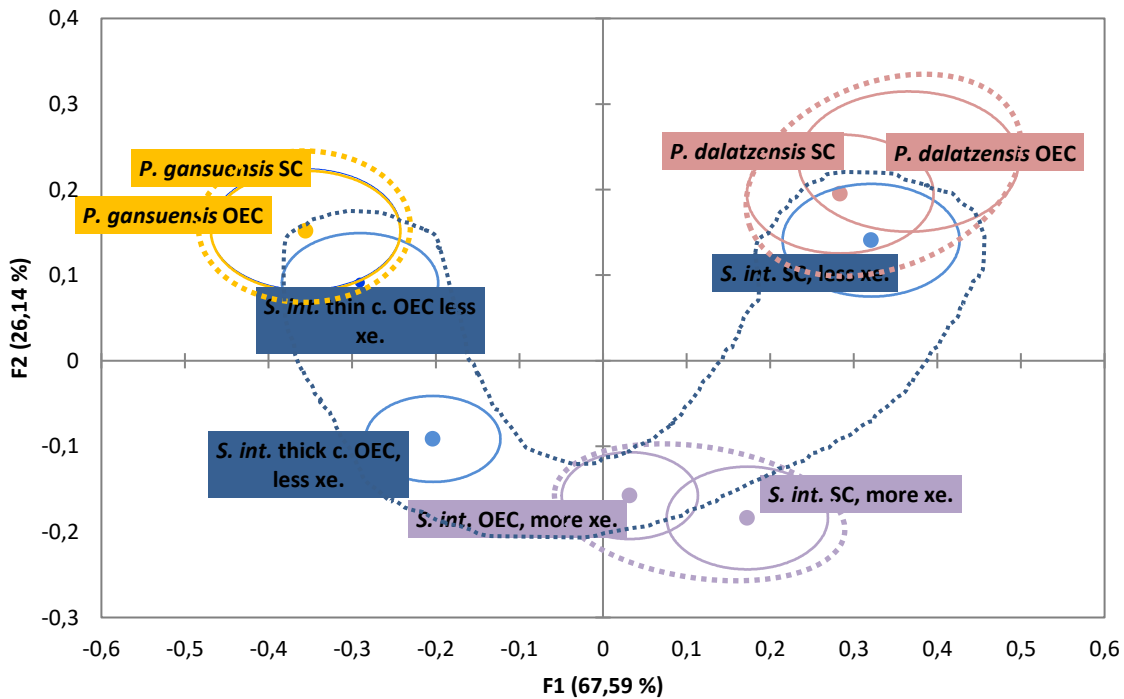


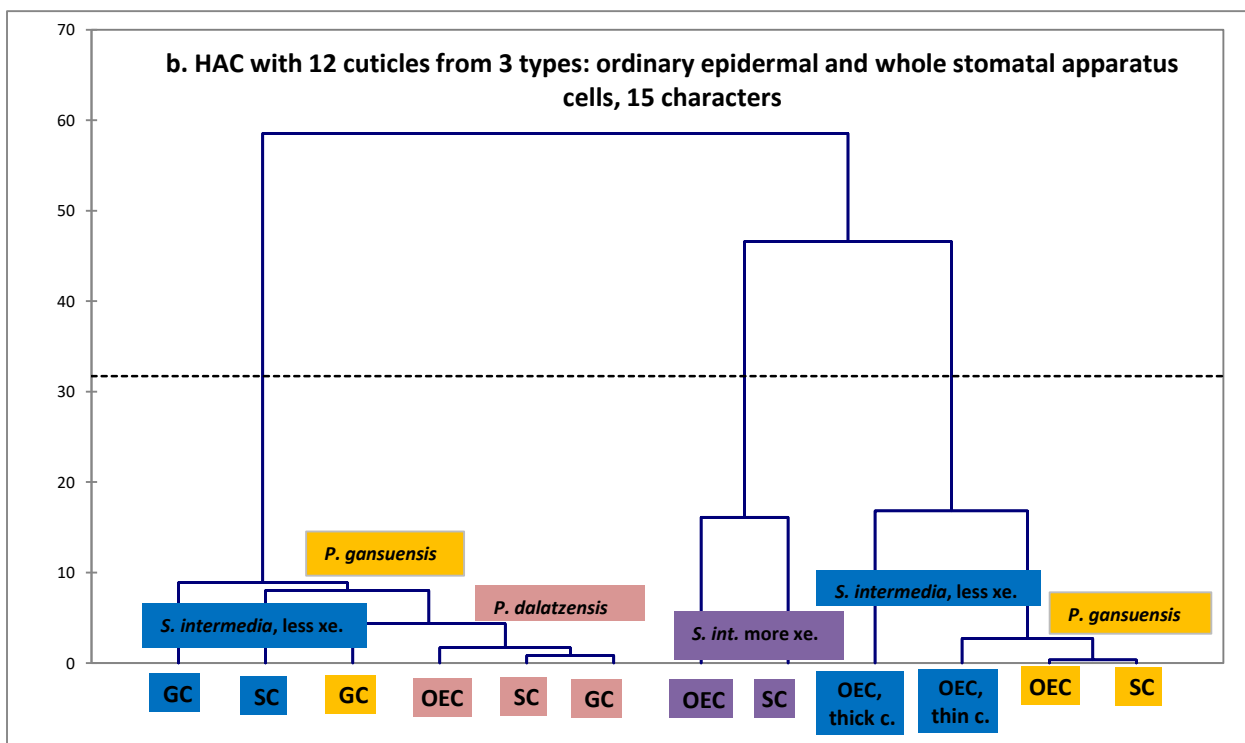
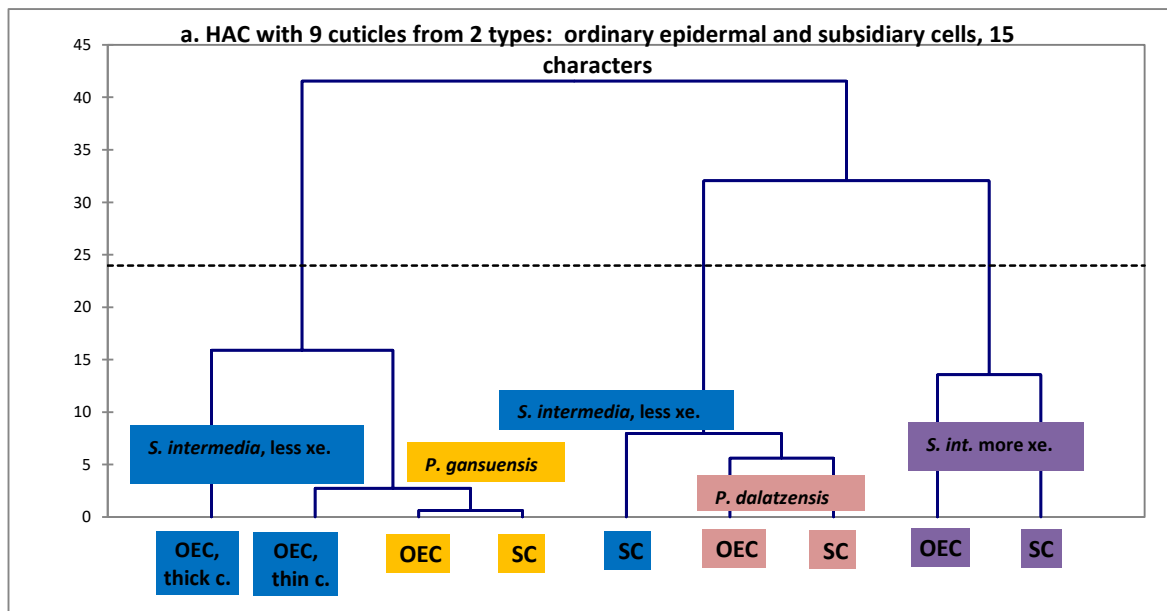


a. Contribution of the 15 characters, 93,72 % of information

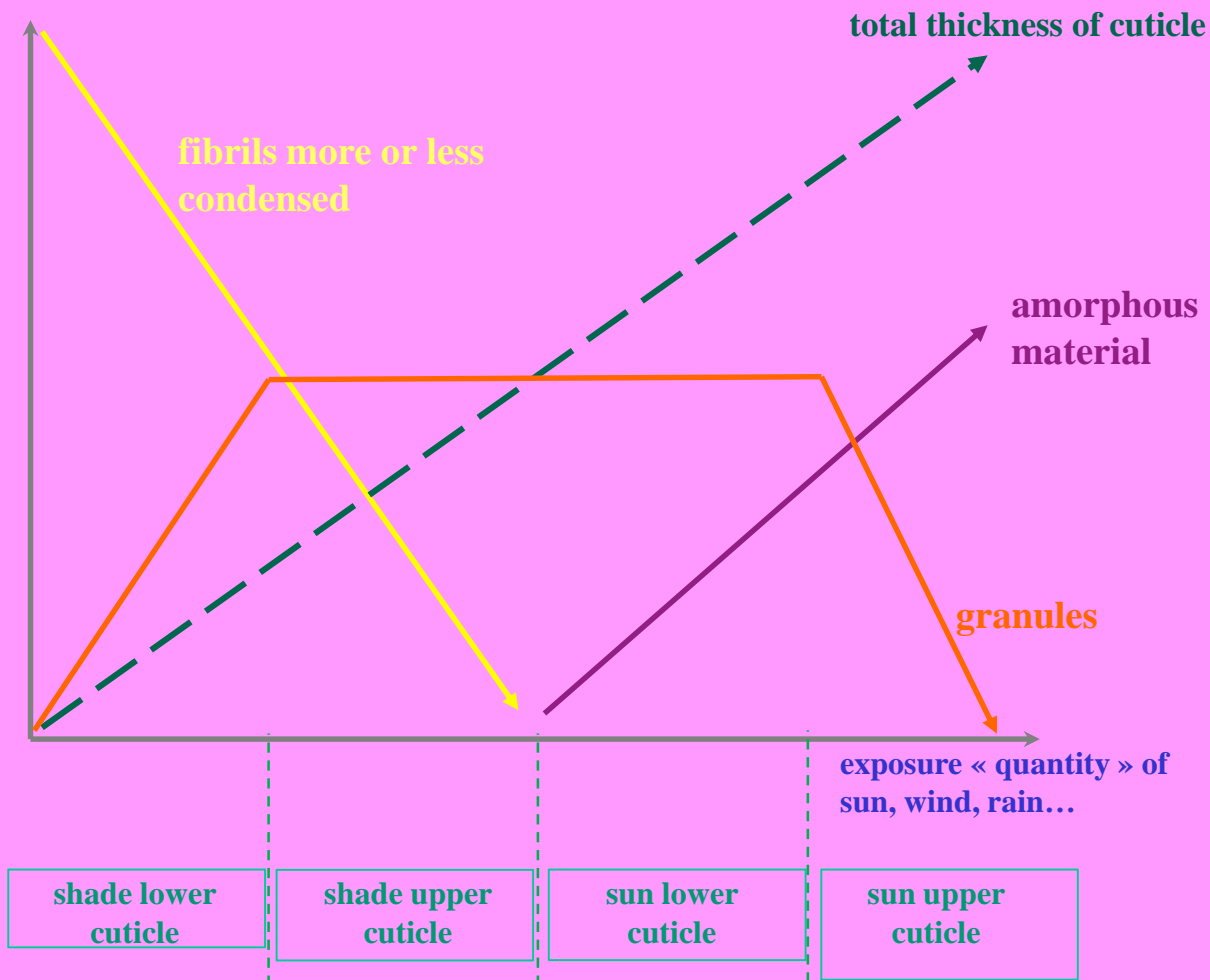


b. The 9 cuticles from 4 taxa belonging to 2 types: ordinary epidermal and subsidiary cells, 93,72 % of information

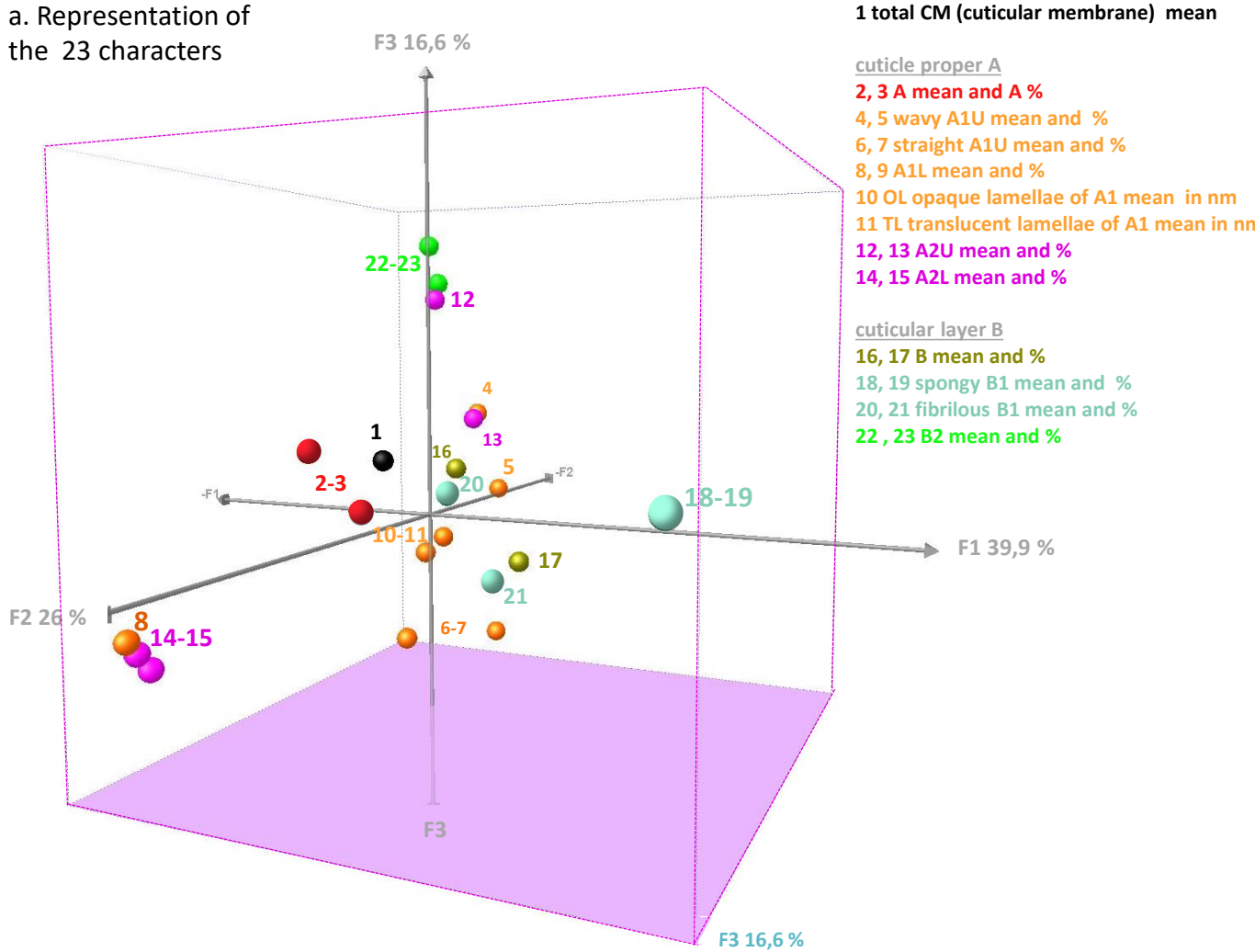




quantity of
cuticle



a. Representation of the 23 characters



b. 13 cuticles of three Coniferales families

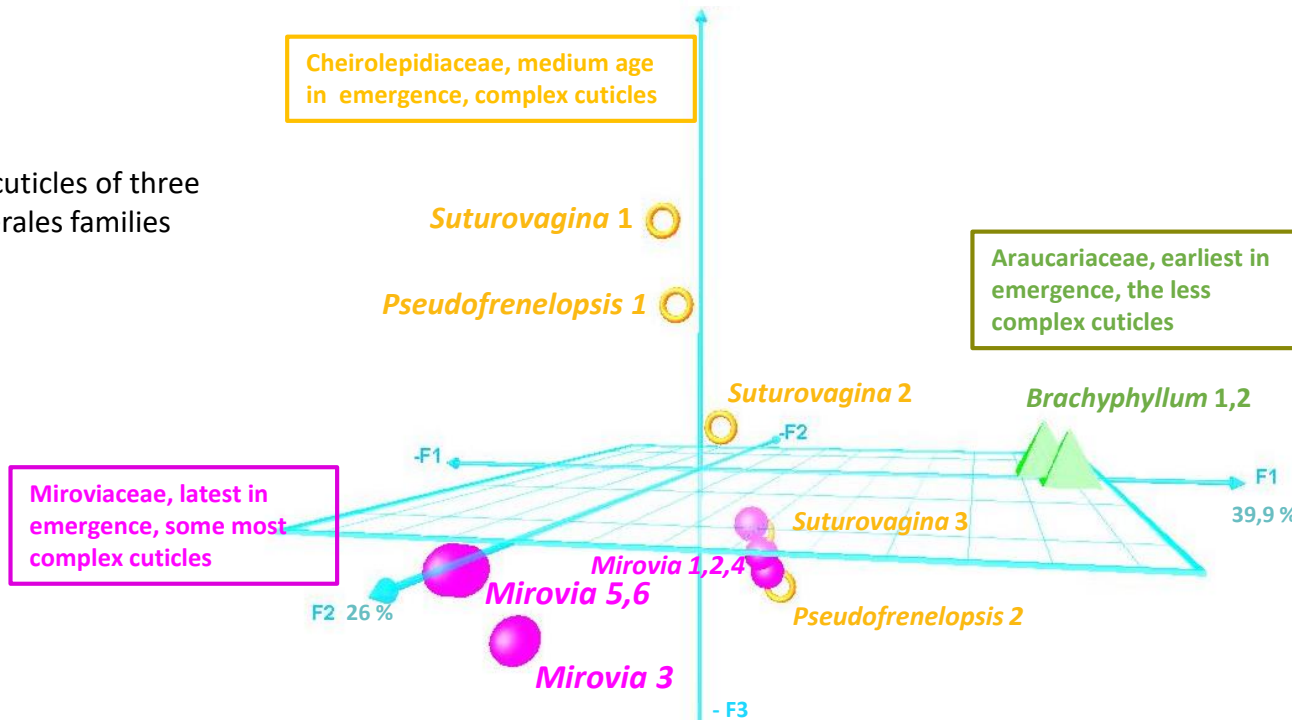


Table 1 selected criteria for various methods

	fixation	postfixation	resin type	staining
Coniferales Cheirolepidiaceae <i>Hirmeriella</i> (Guignard et al. 1998)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 20 min and lead citrate 20 min
Coniferales Cheirolepidiaceae <i>Pseudofrenelopsis</i> (Yang et al. 2009)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Coniferales Cheirolepidiaceae <i>Suturovagina</i> (Mairot et al. 2014)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Coniferales Cheirolepidiaceae <i>Pseudofrenelopsis</i> (Guignard et al. 2017)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Coniferales Cheirolepidiaceae <i>Suturovagina</i> (Yang et al. 2018)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Coniferales <i>Glenrosa</i> Zhou et al. 2000			Spurr and Epon	
Coniferales Podocarpaceae <i>Squamastrobus</i> (Archangelsky and Del Fueyo 1989)		osmium tetraoxide 1%	Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Coniferales Podocarpaceae <i>Morenoa</i> (Del Fueyo et al. 1990)		osmium tetraoxide 1%	Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Coniferales Podocarpaceae <i>Squamastrobus</i> (Barale et al. 1992)			Spurr	potassium permanganate 1,5% 15 min, uranyl acetate 2% 5 min and lead citrate 2-3 min
Coniferales Taxodiaceae <i>Athrotaxis</i> (Villar de Seoane 1998)			Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Coniferales Cheirolepidiaceae <i>Tomaxellia</i> (Villar de Seoane 1998)			Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Coniferales Miroviaceae <i>Mirovia</i> (Nosova et al. 2016)		osmium tetraoxide 2%	Epon- araldite	lead citrate 5 min
Coniferales Araucariaceae <i>Araucaria</i> (Del Fueyo and Archangelsky 2002)		osmium tetraoxide 2%	Spurr	lead citrate 1 min and uranyl acetate 10 min
Coniferales Araucariaceae affinity <i>Brachyphyllum</i> (Carrizo et al. 2019)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Cycadales <i>Ticoa</i> (Archangelsky et al. 1986)		osmium tetraoxide 1%	Spurr	potassium permanganate 5% 10-30 min, uranyl acetate 2% 5-10min or lead citrate 1-5min
Cycadales <i>Pseudoctenis</i>			Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s

(Archangelsky et al. 1995)				
Cycadales <i>Cycas</i> (Bajpai 2000)	glutaraldehyde 4%	osmium tetraoxide 2%	Spurr	uranyl acetate and lead citrate
Cycadales <i>Mesosingeria, Ticoa</i> (Villar de Seoane 2005)		osmium tetraoxide	Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Cycadales <i>Restrepophyllum</i> (Passalia et al. 2010)	glutaraldehyde	osmium tetraoxide 2%	Spurr	lead citrate 1min and uranyl acetate 10 min
Pteridospermales <i>Pachypteris</i> (Baldoni and Barale 1996)			Spurr	potassium permanganate 1% 10 min, uranyl acetate 2% 5 min, lead citrate 2-3 min
Corystospermaceous taxa <i>Dicroidium</i> (Maheshwari and Bajpai 1996a)	glutaraldehyde 4%	osmium tetraoxide 2%	Spurr	uranyl acetate 15 min and lead citrate 5 min
Pteridospermales <i>Pachypteris, Komlopteris</i> (Bajpai and Maheshwari 2000)	glutaraldehyde 4%	osmium tetraoxide 4%	Spurr	uranyl acetate and lead citrate
Pteridospermales <i>Komlopteris</i> (Guignard et al. 2001)	paraformaldehyde 4%	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 or 20 min and lead citrate 20 min
Corystospermales <i>Pachypteris</i> (Guignard et al. 2004)	paraformaldehyde	osmium tetraoxide	Epon	
Corystospermales <i>Zuberia</i> (Martinez et al. 2019)		osmium tetraoxide 2%	Spurr	potassium permanganate 5-10 min, uranyl acetate 30 sec
Bennettitales <i>Zamites, dictyozamites, Ptilophyllum, Otozamites, Cycadolepis</i> (Barale and Baldoni 1993)		potassium permanganate 1%	Spurr	potassium permanganate 1% 5 min, uranyl acetate 5% 10 min and lead citrate 2 min
Bennettitales <i>Otozamites</i> (Villar de Seoane 1999)			Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Bennettitales <i>Otozamites, Pterophyllum, Ptilophyllum, Cycadolepis</i> (Villar de Seoane 2001)			Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Bennettitales <i>Dictyozamites, Otozamites, Zamites, Ptilophyllum, Pterophyllum, Cycadolepis, Williamsonia</i> (Villar de Seoane 2003)		osmium tetraoxide	Spurr	potassium permanganate 5-10 min and uranyl acetate 30 s
Bennettitales <i>Ptilophyllum</i> (Carrizo et al. 2019a)	technique of Del Fueyo and Archangelsky 2005	Osmium tetraoxide 2%	Spurr	lead citrate 1 min, uranyl acetate 10 min
Ginkgoales <i>Ginkgoites</i> (Villar de Seoane 1997)			Spurr	
Ginkgoales <i>Ginkgoites</i> (Del Fueyo et al. 2013)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Ginkgoales <i>Ginkgoites</i> (Guignard et al. 2016)	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min
Ginkgoales <i>Pseudotorellia</i> (Nosova and Yakovleva 2019)	paraformaldehyde - glutaraldehyde	osmium tetraoxide 2%	Epon-araldite	lead citrate 5 min
Ginkgoales <i>Baiera</i>	paraformaldehyde	osmium tetraoxide 1%	Epon	uranyl acetate 7% 15 min and lead citrate 20 min

(Guignard et al. 2019)				
Ginkgoales <i>Sphenobaiera</i> (Wang et al. 2005)		osmium tetraoxide	Epon	uranyl acetate and lead citrate

