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Structural organization of epidermis in leaves of extant species of *Gnetum* L. (Gnetales) and Middle Jurassic Bennettitales

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Abstract

An attempt to assess the identification and evaluation of correlations between the epidermal characters of leaves in Bennettitales and the comparison of the results with the structural organization of leaves of extant plants has been carried out. Epidermis of four species of *Gnetum* L. and four species of Jurassic Bennettitales (*Nilssoniopteris* Nathorst and *Ptilophyllum* Morris) was studied. The coefficients of variation for epidermal characters *Gnetum* range from 6.4 to 24.0 %, *Ptilophyllum* — 15.7–63.5 % and *Nilssoniopteris* — 18.0–39.9 %. In both groups of plants, the sinuosity of the tangential cell walls of the epidermal cells in the upper and lower epidermis is a stable character (Cv ≤ 18.0 %). In the *Ptilophyllum* and *Gnetum*, the length of stomata demonstrates a low level of variability (Cv ≤ 16.8 %). A significant range of the coefficients of variation in both *Gnetum* and Bennettitales show the number of epidermal cells per 1 mm^2 of the upper and lower epidermis (17.5 % ≤ Cv ≤ 31.9 %), the area of the epidermal cells in the upper and lower epidermis (21.2 % ≤ Cv ≤ 63.5 %) and the number of stomata per 1 mm^2 of epidermis (29.3 % ≤ Cv ≤ 39.9 %). Similarities in the correlation structure of the epidermal characters are revealed in correlations between sinuosity of the tangential cell walls of the epidermis, the number of stomata per 1 mm^2 and their size; the length of stomata and the number of epidermal cells; stomatal index and the number of epidermal cells of the epidermis. In *Gnetum*, the number of differentiated stomata correlates with the number of aborted stomata. In *N. angustifolia* and *P. caucasicum*, the number of stomata correlates with the number of papillae per 1 mm^2. Similarities in correlational structure of epidermis in *Nilssoniopteris*, *Ptilophyllum,* and *Gnetum* could be ecological adaptations or ontogenetic characters, such as the development of stomata that has been described by other researchers.

Keywords: paleobotany, plant fossils, Bennettittales, *Gnetum*, leaf, structural organization

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Introduction

Gnetales and Bennettitales are two taxa of gymnosperms whose evolution occurred in parallel (Crane, 1996; Donoghue and Doyle, 2000; Friis et al., 2007). Both taxa appeared at approximately the same time, at the beginning of the Mesozoic era: Gnetales — at the Permian-Triassic boundary (Wang, 2004), Bennettitales — at Late Triassic (Krassilov and Bugdaeva, 1988). They developed under similar ecological conditions (Crane, 1996). In this regard, both groups revealed some common structural characters. For example, bisexual strobili (Carmichael and Friedman, 1996; Friedman, 1998; Donoghue and Doyle, 2000; Kiritchkova and Nosova, 2012): The male cones of extant Gnetales have nonfunctional ovules, which are considered by some authors as traces of the past bisexuality in ancestral plants (Haycraft and Carmichael, 2001). Another example is the similarity in the structure of epidermis and stomata. Thomas and Bancroft (Thomas and Ban-

croft, 1913) described the epidermis of Bennettitales and found that the stomata are paracytic. This stomatal type is not found in cycads, to which the Bennettitales were originally assigned, and among other gymnosperms it is known only in *Gnetum* and *Welwitschia* (Florin, 1931, 1933; Nautiyal, Singh, and Pant, 1976) and *Podozamites* (Shi et al., 2017), but it is also widely distributed among flowering plants (Donoghue and Doyle, 2000).

Florin (Florin, 1931, 1933) described the development of stomata in Bennettitales: The mother cell of the stomata in the protoderm divides unequally twice, leading to the formation of subsidiary cells, and as a result of the final equal division, guard cells of the stomata are formed. Thus, the origin of subsidiary and guard cells is the same — from one meristemoid. However, epidermal cells at the poles of the stomata develop from neighboring protodermal cells — they have a different origin than subsidiary and guard cells. This shows the similarity in the development of the stomatal apparatus in leaves of Bennettitales and Gnetum species (Rudall and Bateman, 2019).

Indian researchers (Nautiyal, Singh, and Pant, 1976), who studied in detail the histogenesis of epidermis in *Gnetum*, found that the paracytic stomata has a dual origin: guard cells and subsidiary cells are formed from one meristemoid — mesogene origin, and neighboring epidermal cells at the poles of stoma arise from other cells of protoderm — perigene origin (Rudall and Bateman, 2019). According to the classification of D. D. Pant (Pant, 1965), this is a variant of meso-perigenic stomata.

Angiosperms have a wide range of ways of origin of paracytic stomata, depending on whether the cells surrounding the stomata develop from the same mother cell as the guard cells, or from other protodermal cells, or also have a dual origin (Pant, 1965).

Stomatal traits have played a significant role in inferring seed-plant phylogeny, and the presence of paracytic stomata in Bennettitales represents a synapomorphy linking them with angiosperms in most analyses. Rudall and Bateman (Rudall and Bateman, 2019) conclude that the stomata of *Gnetum* represent by far the most likely analogue for stomatal development in Bennettitales.

The leaf epidermis of Bennettitales is well-investigated (Florin, 1931, 1933; Doludenko and Svanidze, 1969; Kiritchkova and Nosova, 2012). However, studies comparing quantitative characters and revealing the structural organization of epidermis of extinct plants using multivariate statistics methods have not been carried out. We performed an attempt to adapt a method of principal component analysis for the leaves of fossil plants, which could be useful in traditional studies of taxonomy and phylogeny and become an alternative to molecular taxonomy. Due to this analysis, it is possible to compare the structures of extinct and extant plants.

The purpose of our study was to estimate the common variability and correlations between the structural characters of the epidermis in extinct species of Bennettitales (genera *Nilssoniopteris* Nathorst and *Ptilophyllum* Morris) and extant species of *Gnetum,* and to evaluate common characters and differences or similarities in the structural organization of leaves.

Fossilized leaves of Bennettitales differ from each other, ranging from a few centimeters to more than a meter in length. The leaves are predominantly unipinnate. Rarely, the leaves are simple. The species are described according to the epidermal characters of leaves, in particular, the structure of the stomatal apparatus, the shape of the cells, the presence of papillae and trichomes (Rudall and Bateman, 2019). Two genera of Jurassic Bennettitales were the objects of our study: *Nilssoniopteris* with a simple leaf blade and *Ptilophyllum* with a leaf blade dissected into segments.

The cuticles, on which imprints of cells of epidermis are present, are well-preserved in fossils. This makes it possible not only to describe the structure accurately, but also to evaluate the quantitative characters, and to try to identify, at least partially, some characters of the structural organization of leaves in Bennettitales. For example, to assess the correlations between the structural characters of leaf epidermis. The results obtained for epidermis of extinct plants were compared with similar data for extant *Gnetum* via cuticular analysis (Kerp, 1990).

In recent decades, many fossils of Gnetales have been found (Krassilov and Bugdaeva, 1988; Rydin and Friis, 2010; Friis et al., 2007), but there are no species described on epidermal characters (Crane, 1996). However, the epidermis of reproductive structures and shoots has been described (Rydin and Friis, 2010). According to molecular data, the *Gnetum* arose approximately in the Eocene or Middle Miocene. Of all the extant Gnetales, only *Gnetum* is not represented in the fossil record (Won and Renner, 2006), possibly due to the fact that their leaves are similar to the leaves of flowering plants, and it is difficult to distinguish them in fossils by morphological and anatomical characters (Crane, 1996). Only *Protognetum* from Middle Jurassic is known, which combines the vegetative characters of *Ephedra* with reproductive characters of *Gnetum* (Yang, Xie, and Ferguson, 2017). Gambaryan and Kuznetsov (Gambaryan and Kuznetsov, 2021), creating a model of a hypothetical ancestor of flowering plants, suggest that the hypothetical plant of Gnetales from the Jurassic could resemble in appearance the modern tree species *Gnetum gnemon* L.

Unlike other modern genera (leaves of *Ephedra* L. are reduced, and *Welwitschia* have only two leaves, which grow constantly due to intercalary meristem), species of *Gnetum* have simple leaves, which are ideal model objects to compare with the leaves of Bennettitales.

Materials and methods

Plant material. Fossils of Bennettitales were taken from collection No. 1073, Laboratory of Paleobotany, BIN RAS. The samples were collected by G. V. Delle (1980) in Georgia, river Barula, Tsesi village. Dating: Middle Jurassic (Callovian) (Doludenko and Svanidze, 1969).

The cuticle of 73 specimens belonging to 2 genera was studied. Ianina O. Bogdanova made the determination.

Nilssoniopteris Nathorst — 2 species:

N. angustifolia Doludenko, 9 samples: BIN 1497, 1498, 1521, 1536, 1538, 1540, 1541, 1560, 1564;

N. longifolia Doludenko, 16 samples: BIN 1509, 1510, 1519, 1520, 1525, 1526, 1527, 1528, 1530, 1532, 1537, 1550, 1556, 1557, 1562, 1563.

Ptilophyllum Morris — 2 species:

P. caucasicum Doludenko et Svanidze, 20 samples: BIN 1573, 1586, 1593, 1595, 1597, 1598, 1599, 1608, 1613, 1619, 1620, 1621, 1623, 1639, 1642, 1649, 1650, 1651, 1656, 1683;

P. okribense f. ratchense Doludenko et Svanidze, 28 samples: BIN 1568, 1572, 1581, 1584, 1589, 1592, 1600, 1601, 1602, 1604, 1606, 1607, 1615, 1617, 1622, 1631, 1632, 1635, 1637, 1638, 1646, 1647, 1648, 1657, 1658, 1672, 1679, 1680.

Gnetum L.: upper and lower epidermis from 76 mature leaves belonging to 4 species was studied.

G. gnemon L. — 24 leaves: 21 leaves were collected in greenhouse No. 20 of the Botanical Garden BIN RAS, 3 leaves were taken from herbarium LE, collected in Vietnam: LE 01068309, LE 01068330.

G. gracilipes C. Y. Cheng — 16 leaves. The samples were collected in Yunnan Province, China (N 21.63, E 101.44, 2018).

G. latifolium Blume — 11 leaves. The material was taken from the herbarium of the BIN RAS (LE), collected in Yunnan Province and Hainan Island (China), Vietnam: LE 01068250, LE 01068251, LE 01068253, LE 01068254, LE 01068256, LE 01068257, LE 01068259, LE 01068260, LE 01068262, LE 01068263, LE 01068301.

G. montanum Markgr. — 25 leaves: 21 leaves were collected in greenhouse No. 20 of the Botanical Garden BIN RAS and 4 leaves were taken in LE herbarium, collected in Laos and Vietnam: LE 01068323, LE 01068326, LE 01068327.

Material selection. *Gnetum:* fragments of epidermis from the middle part of the leaf blade, located between the main vein and the leaf margin, were studied. Living material was fixed in 70° ethanol. Bennettitales: the fossil material was taken from the middle part of a whole leaf blade (*Nilssoniopteris*), or from the middle part of a leaf segment or a whole segment (*Ptilophyllum*).

Herbarized material. Herbarized leaf fragments were placed in a mixture of glycerol, distilled water and ethanol (70°) in a ratio of 1 : 1 : 1 in a thermostat (temperature 50 °C). The material was washed in distilled water and used for further research.

Maceration of epidermis. Fragments of the leaves of Bennettitales were kept in hydrofluoric acid (HF). Fragments of the leaves of gnetums and Bennettitales were placed in a solution of $KClO_3$ and HNO_3 (Yang, Xie, and Ferguson, 1976). Then they were washed with distilled water and placed in a KOH solution. After that, the fragments were again washed with distilled water. Using a dissecting needle, the upper and lower epidermis of gnetums and the cuticle of Bennettitales were separated. The separated epidermis and cuticle were placed in 70° ethanol. Subsequently, fragments of the epidermis of gnetums were stained with safranin. Fragments of the epidermis and the fossil cuticle were poured into a glycerol-gelatin.

Light and scanning electron microscopy. Viewing and photographing of the slides were carried out using a Leica EZ4 binoculars, Leica DM500 microscopes, Leica DM1000 microscope and Leica EC3 digital camera (Germany). The obtained data were processed in the ImageJ. Photographing of the fossilized leaves of Bennettitales was carried out using a Canon EOS 770 camera.

For scanning electron microscopy, the leaf fragments were dehydrated in a series of ethanol of increasing concentrations (20°, 50°, 70°, 80°, 90°, 96°, 100°). Then, they were dipped into mixtures of acetone and ethanol (100°), acetone and isoamyl acetate and isoamyl acetate only. Dehydrated specimens underwent critical point drying using liquid carbon dioxide (CO_2). Dried objects were applied to a stub and sprayed with ions of gold. We used a scanning electron microscope JSM-6390LA (Japan) for the examination of the prepared specimens.

Description of the epidermis. The cuticular analysis was used for description of fossils (Kerp, 1990). A feature of the inner surface of the cuticle of fossil plants is that the surface of the epidermis is imprinted on it: a sculpture of epidermal cells, stomata and trichomae. Accordingly, it becomes possible to analyze the structure of epidermis of extinct plants and compare it with the epidermis of extant plants according to key characters.

For description of qualitative characters of the epidermis, the classification of Vasiliev (Vasiliev, 1988) was used; for description of shapes, sinuosity of tangential walls and projections of the epidermal cells the classification of Zakharevich (Zakharevich, 1954) was used.

Statistical methods. The obtained data were processed in the Statistica 8.0. The coefficient of variation

(Cv) was calculated to determine the general variability of the characters. The assessment of the level of common variability of characters was carried out in accordance with the classification of Mamaev (Mamaev, 1973).

The coefficient of sinuosity of the tangential cell walls of the epidermal cells in upper epidermis was calculated using the formula (Vasiliev, 1988):

$$\text{Sin\_upp} = \frac{\text{Per\_upp}}{\text{Supp}},$$

Sin_upp — sinuosity of epidermal cells in lower epidermis; **Per_upp** — perimeter of epidermal cells in upper epidermis, μm; **Supp** — area of epidermal cells in upper epidermis, μm^2. The sinuosity of epidermal cells in lower epidermis was calculated in the same way.

Correlations between characters were determined using principal component analysis (Kendall and Stewart, 1976). For statistical methods, 13 characters that describe the structure of epidermis were selected. They are listed in tables 1 and 2.

Table 1. Statistical characteristics of the characters of the leaf structure for *Gnetum* species

Characters	*G. gnemon*					*G. gracilipes*				
	M	σ	m_x	X_{min}	X_{max}	M	σ	m_x	X_{min}	X_{max}
Ncell_upp	2875	361	587	2166	3425	1537	375	384	1120	2567
Supp	319	49	65	249	409	732	163	183	380	961
Per_upp	99	10	20	79	125	144	21	36	92	171
Sin_upp	1.39	0.15	0.28	1.08	1.66	1.34	0.07	0.33	1.19	1.42
Ncell_low	2401	370	490	1609	3040	1414	480	475	972	1856
Slow	495	121	101	367	814	873	247	218	432	1273
Per_low	119	19	24	92	153	160	26	40	103	196
Sin_low	1.34	0.14	0.27	1.05	1.56	1.37	0.06	0.34	1.25	1.50
Nstom	296	84	60	188	543	205	68	51	135	438
Ndstom	202	52	41	127	295	182	48	46	122	336
Lstom	25	2	5	23	30	29	1	7	27	32
Kstom	7.12	1.65	1.45	4.88	11.76	7.50	0.41	1.88	6.55	8.00
	G. latifolium					*G. montanum*				
Ncell_upp	1231	335	371	978	2179	906	188	181	424	1312
Supp	819	146	247	465	1061	1112	325	222	718	2393
Per_upp	120	24	36	77	162	176	36	35	117	299
Sin_upp	1.13	0.08	0.34	1.01	1.24	1.32	0.12	0.26	1.06	1.53
Ncell_low	1101	73	332	990	1250	970	145	194	521	1187
Slow	1061	171	320	714	1293	1240	335	248	809	2457
Per_low	146	27	44	90	192	193	30	39	148	285
Sin_low	1.21	0.09	0.36	1.07	1.33	1.39	0.11	0.28	1.22	1.65
Nstom	168	43	51	121	285	162	29	31	98	232
Ndstom	151	36	46	118	248	137	27	27	72	213
Lstom	32	2	10	28	36	30	2	6	27	33
Kstom	10.66	1.38	3.21	9.09	14.18	10.08	1.04	2.02	7.88	12.56

Notes: M — mean; m_x — standard error of the mean; σ — standard deviation; X_{min} — minimum; X_{max} — maximum.

Characters: **Ncell_upp** — number of epidermal cells per 1 mm^2 of upper epidermis; **Supp** — area of epidermal cells in upper epidermis, μm^2; **Per_upp** — perimeter of epidermal cells in upper epidermis, μm; **Sin_upp** — sinuosity of epidermal cells in lower epidermis; **Ncell_low** — number of epidermal cells per 1 mm^2 of lower epidermis; **Slow** — area of epidermal cells in the lower epidermis, μm^2; **Per_low** — perimeter of epidermal cells in lower epidermis, μm; **Sin_low** — sinuosity of epidermal cells in lower epidermis; **Nstom** — number of stomata per 1 mm^2 of epidermis; **Ndstom** — number of differentiated stomata per 1 mm^2 of epidermis; **Lstom** — length of stomata, μm; **Kstom** — stomatal index, %.

Results and discussion

Morphological and anatomical characteristics of the mature leaves of *Gnetum* species. The studied leaves are simple, divided into blade and petiole, small, with a blade area of 11.5–18 cm^2 (*G. gnemon, G. montanum*) or medium size, with a blade area of 40–152 cm^2 (*G. gnemon, G. gracilipes, G. latifolium, G. montanum*) (Fig. 1A, E; Fig. 2A, E).

Leaves are hypostomatic. In the upper epidermis above large veins of *G. gnemon, G. gracilipes, G. montanum* occasional stomata are noted.

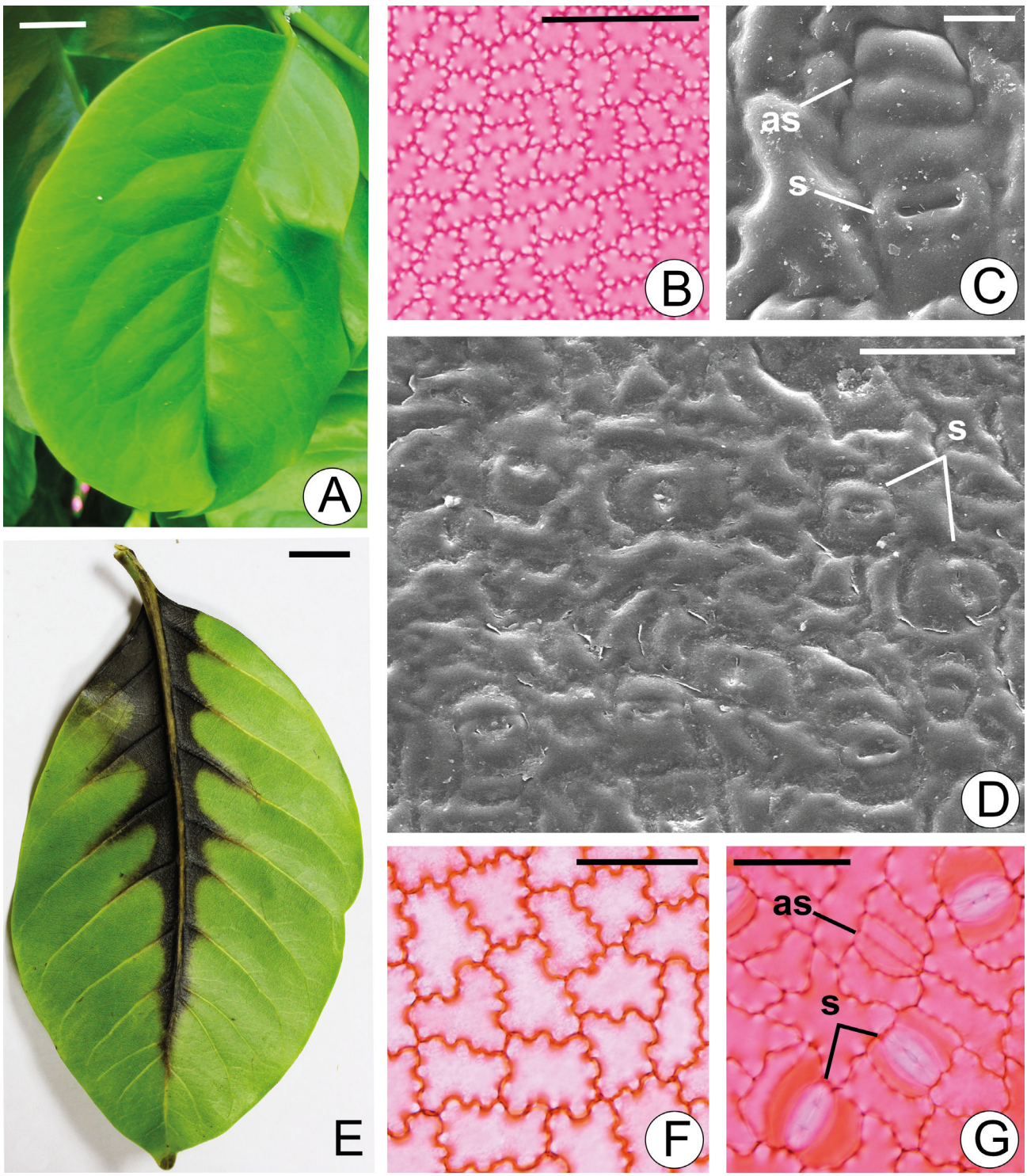

Fig. 1. Epidermal structure of *Gnetum* leaves. *G. gnemon*: *A* — leaf; *B* — fragment of upper epidermis; *C* — fragment of lower epidermis with differentiated (*s*) and aborted (*as*) stomata; *D* — fragment of lower epidermis with stomata. *G. gracilipes*: *E* — leaf; *F* — fragment of upper epidermis; *G* — fragment of lower epidermis with differentiated and aborted stomata.

Scale bar: *A, E* — 1 cm; *B, D, E, G* — 50 μm; *C* — 10 μm.

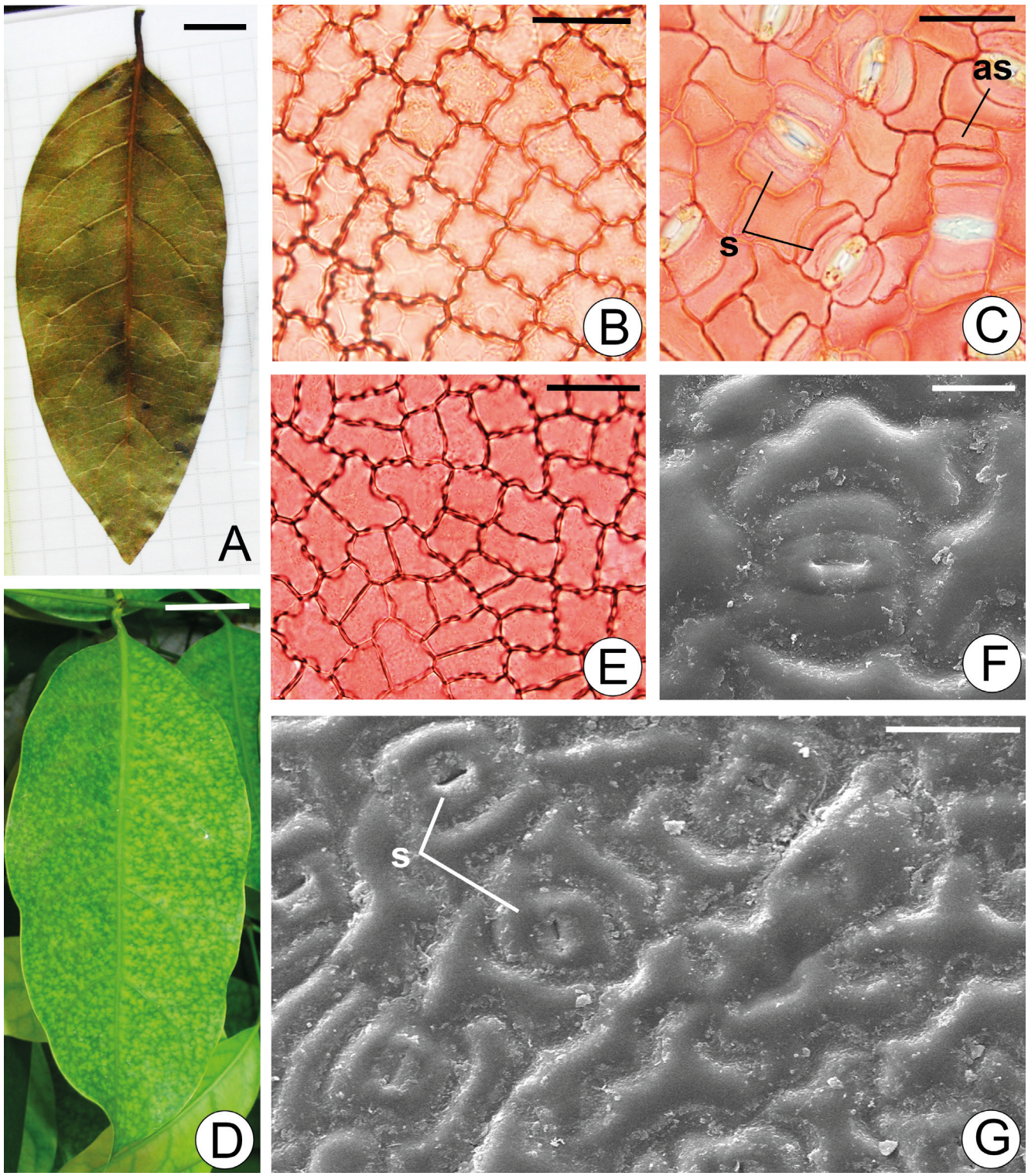

Fig. 2. Epidermal structure of *Gnetum* leaves. *G. latifolium*: *A* — leaf; *B* — fragment of upper epidermis; *C* — fragment of lower epidermis with differentiated (*s*) and aborted (*as*) stomata. *G. montanum*: *D* — leaf; *E* — fragment of upper epidermis; *F* — stoma in lower epidermis; *G* — fragment of lower epidermis with stomata.

Scale bar: *A, D* — 1 cm; *B, C, E* — 50 μm; *F* — 10 μm; *G* — 30 μm.

The upper epidermis (Fig. 1B, F; Fig. 2B, E; Table 1) consists of very small-sized cells (~3400 cells per 1 mm^2) in *G. gnemon*; small-sized cells (1600–2500 cells per 1 mm^2) in *G. gnemon* and *G. latifolium*; medium-sized cells (900–1600 cells per 1 mm^2) in *G. gracilipes, G. latifolium* and *G. montanum*; large-sized cells (400–900 cells per 1 mm^2) in *G. gracilipes* and *G. montanum*.

The shape of the epidermal cells is diverse. The projection of the cell area above the mesophyll is square, rectangular or polygonal; above small and large veins the

projection is rectangular or square. The outlines are rectilinear, wavy, sinuous or sinuous-wavy. The coefficient of sinuosity of the tangential cell walls ranges from 1.006 to 1.66. Minimal values (≤ 1.1) are in the epidermis of *G. gnemon, G. latifolium*, and *G. montanum*; maximal values (1.66) are in the epidermis of *G. gnemon*.

The lower epidermis (Fig. 1C, D, F, G; Fig. 2C, D, F, G) consists of small-sized cells (2400–3040 cells per 1 mm^2) in *G. gnemon*; medium-sized cells (1350–1800 cells per 1 mm^2) in *G. gnemon, G. gracilipes* and *G. latifolium*; large-sized cells (1000–1250 cells per 1 mm^2) in *G. gracilipes, G. latifolium* and *G. montanum*; very large-sized cells (~520 cells per mm^2) in *G. montanum*.

The shape of the epidermal cells of the lower epidermis is also diverse. The projection of their area above the mesophyll is elongated, polygonal or rectangular; above small and large veins it is rectangular. The outlines are wavy, large-wavy, rectilinear, sinuous, sinuous-wavy. The coefficient of sinuosity of tangential cell walls ranges from 1.05 to 1.65. Minimal values (≤ 1.1) are in the epidermis of *G. gnemon* and *G. latifolium*; maximal values (1.65) are in *G. montanum*.

There are very few stomata on the surface of epidermis (~70 stomata per 1 mm^2) in *G. montanum*; few stomata (110–120 stomata per 1 mm^2) in *G. gracilipes, G. latifolium* and *G. montanum*; average number of stomata (210–230 stomata per mm^2) in *G. gnemon, G. gracilipes, G. latifolium* and *G. montanum*; many stomata (275–330 stomata per 1 mm^2) in *G. gnemon, G. gracilipes* and *G. latifolium*. The stomata are arranged irregularly. In the areas of the epidermis above large veins, occasional stomata are noted.

The stomatal Index is very low (4.9 %) in the epidermis of *G. gnemon*; low (6.5–11.8 %) in *G. gnemon, G. gracilipes, G. latifolium* and *G. montanum*; medium (12.6–14.2 %) in *G. latifolium* and *G. montanum*.

The predominant type of stomata is paracytic (Fig. 1C, D, G; Fig. 2C, F, G). There were noted laterocytic, anomocytic, encyclocytic, hemiparacytic types, as well as transitional variants of stomata (for example, transitional to encyclocytic type in *G. gnemon, G. latifolium* and *G. montanum*; transitional to paracytic type in *G. latifolium*; transitional to laterocytic and hemiparacytic types in *G. montanum*). Aborted stomata are noted in the epidermis of *G. gnemon, G. gracilipes*, and *G. latifolium* (Fig. 1C, G; Fig. 2C). In all the studied specimens on the surface of the lower epidermis, cork warts were found. They distributedabove the mesophyll and along large veins.

Morphological and anatomical characteristics of the leaves of Bennettitales

Nilssoniopteris. *N. angustifolia*. The leaves are simple, linear, gradually narrowed at the apex and base (Fig. 3A). The apex is pointed. The petiole is thin. The veins are thin, simple, and dichotomous near the origin of the shaft. The length of the leaves is more than 100 mm; the width is 5.5–23 mm in the middle part.

The upper epidermis (Fig. 3B, Table 2) undivided into costal and intercostal fields, consists of large-sized cells (530–900 cells per 1 mm^2), or medium-sized cells (900–1200 cells per 1 mm^2). There are more or less distinct longitudinal rows of cells. The projection of cell area is square or elongated; outlines are sinuous-wavy. The coefficient of sinuosity of the tangential cell walls ranges from 1.2 to 2.1. Trichomae are absent.

The lower epidermis (Fig. 3C–G) consists of medium-sized cells (1350–1450 cells per 1 mm^2), or large-sized cells (610–1350 cells per 1 mm^2). The epidermis is divided into costal and stomatal fields (Fig. 3E). The projection of the area of the epidermal cells in the stomatal fields is round or elongated; outlines are sinuous-wavy or low-sinuous. In costal fields, cells are arranged in rows. Their projection is rectangular or square; outlines are sinuous-wavy, wide-wavy, low-sinuous. The coefficient of sinuosity of the tangential cell walls ranges from 1.2 to 1.7. Most of the epidermal cells in the lower epidermis, both in stomatal and costal fields, have one central convex-shaped papilla; their imprints are well preserved on the cuticle (Fig. 3C, D, F). The number of papillae varies from 300 to 745 per 1 mm^2. Trichomae are absent.

Leaves are hypostomatic. There are from one to three stomata per width of the stomatal field (Fig. 3E, G). Their apertures are oriented at different angles within the field. Occasional stomata can occur in costal fields and above large veins. The type of stomata is paracytic (Fig. 3F, G). The stomatal index is low (9.5–11 %), medium (11–16 %) or large (16–17.0 %). There are very few (~70 per 1 mm^2), few (100–160 per 1 mm^2), average (160–250 per 1 mm^2), and many stomata (~260 per 1 mm^2) on the epidermal surface. The tangential cell walls of subsidiary cells are even and thick.

N. longifolia. The leaves are simple, linear, narrow, gradually and very slightly narrowed at the apex and base (Fig. 4A). The apex is rounded. The petiole is thin. The veins are thin. The length of the leaves is more than 190 mm; the width is 9.1–24.7 mm in the middle part.

The upper epidermis (Fig. 4B, Table 2) consists of medium-sized cells (900–1300 cells per 1 mm^2), or large-sized cells (440–900 cells per 1 mm^2). The projection of the area of the epidermal cells is rectangular or square, above the veins it is elongated. The outlines are sinuous-wavy, wide-wavy. The coefficient of sinuosity of the tangential cell walls ranges from 1.1 to 1.9. Trichomae are absent.

The lower epidermis (Fig. 4C–G) consists of large-sized cells (510–600 cells per 1 mm^2) or very large-sized cells (600–1100 cells per 1 mm^2). The epidermis is divided into costal and stomatal fields (Fig. 4C). The projection of the area of the epidermal cells in the stomatal

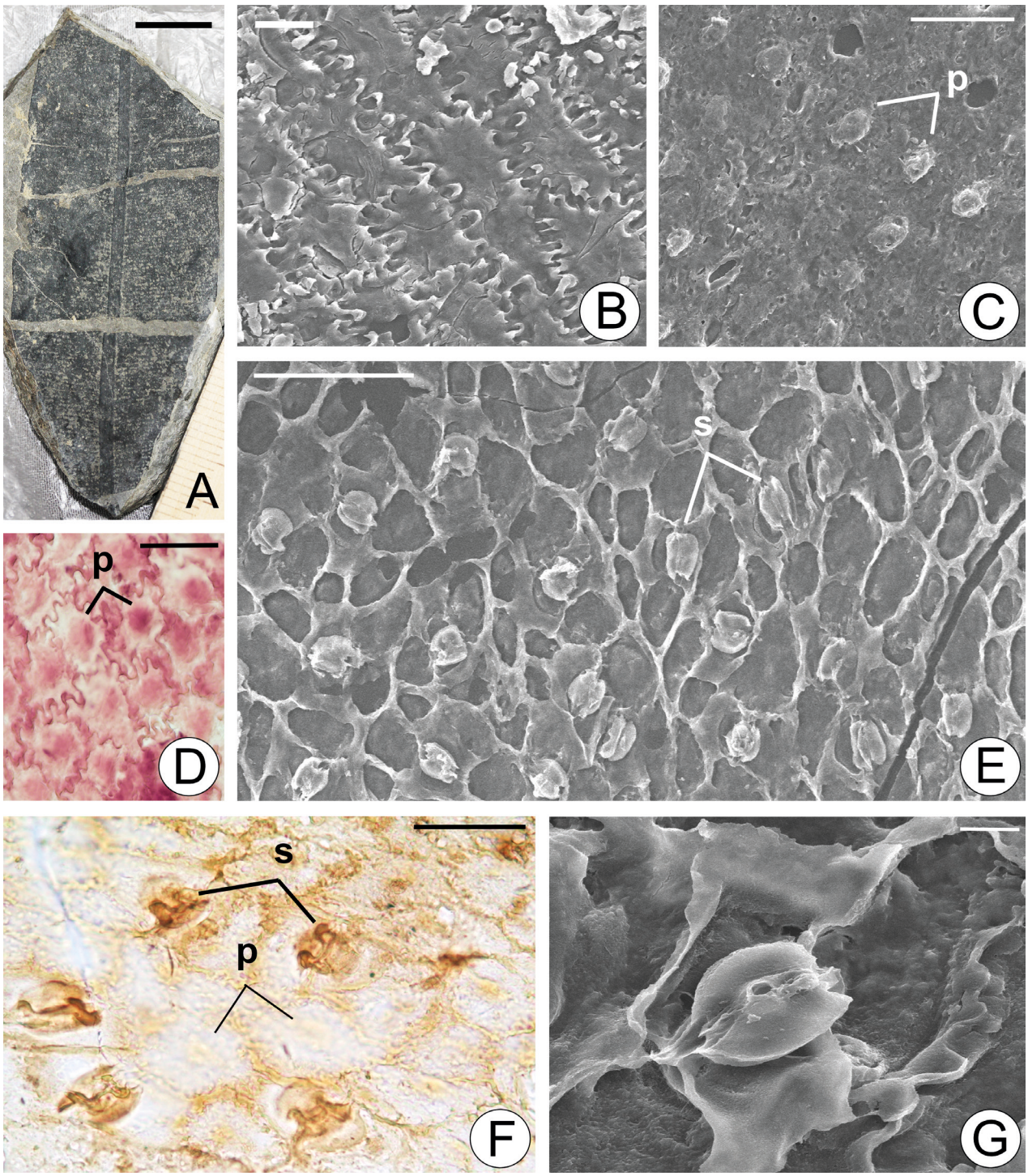

Fig. 3. Leaves of *N. angustifolia*. *A* — fragment of leaf blade, spec. BIN 1560; *B* — cuticle fragment with imprint of upper epidermis, inner view, spec. BIN 1538; *C* — cuticle fragment of lower epidermis with papillae (*p*), external view, spec. BIN 1538; *D* — abaxial side of leaf, a cuticle fragment with papillae, spec. BIN 1538; *E* — cuticle fragment with imprint of lower epidermis with stomata (*s*), inner view, spec. BIN 1564; *G* — abaxial side of leaf, cuticle fragment with stomata, spec. BIN 1536; *7* — stoma, inner view, spec. BIN 1560.

Scale bar: *A* — 1 cm; *B* — 20 μm; *C, D, E* — 50 μm; *F* — 100 μm; *G* — 10 μm.

fields is square or rounded; in costal fields, on the sides, it is square and elongated in the central part. The outlines are sinuous-wavy, wide-wavy. The coefficient sinuosity of the tangential cell walls ranges from 1.1 to 2.4.

The leaves are hypostomatic (Fig. 4C–G). Per width of the stomatal field there are three-four stomata (Fig. 4C). Their apertures are oriented at different angles within the field. Occasional stomata can be found in costal fields or above large veins. On the epidermal surface there are very few (59–100 stomata per 1 mm^2), few (100–160 stomata per 1 mm^2), or average number of stomata (160–250 stomata per 1 mm^2) (Fig. 4C). The type

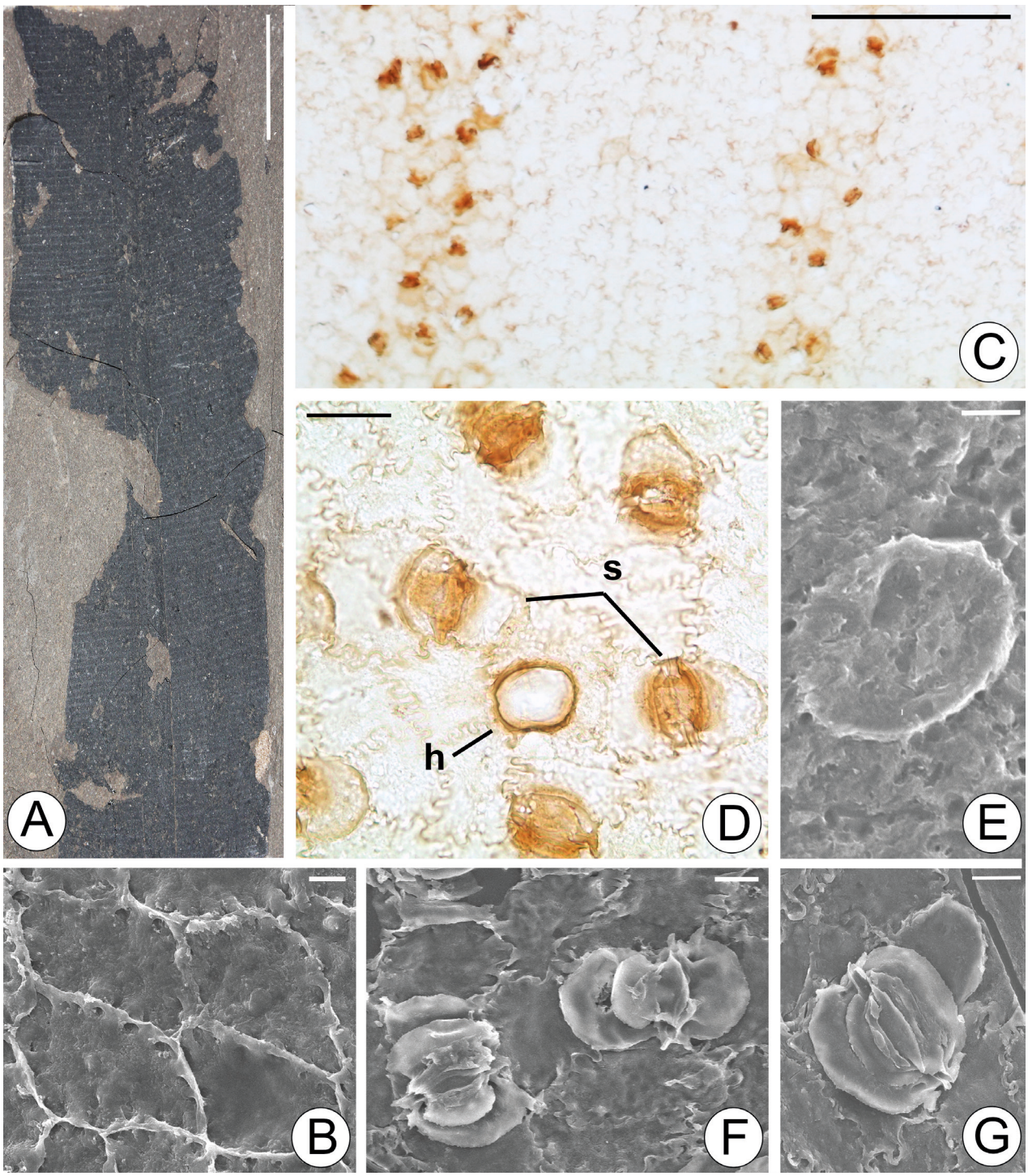

Fig. 4. Leaves of *N. longifolia*. *A* — fragment of leaf blade, spec. BIN 1562; *B* — cuticle fragment with imprint of upper epidermis, inner view, spec. BIN 1525; *C* — abaxial side of leaf, cuticle fragment with stomatal strips, spec. BIN 1528; *D* — abaxial side of leaf, a cuticle fragment with stomata (*s*) and trichome base (*h*), spec. BIN 1527; *E* — trichome base, external view, spec. BIN 1530; *F* — cuticle fragment with imprint of lower epidermis with stomata, inner view, spec. BIN 1509; *G* — stoma, inner view, spec. BIN 1520.

Scale bar: *A* — 1 cm; *B, E* — 10 μm; *C* — 200 μm; *D* — 50 μm; *F, G* — 20 μm.

of stomata is paracytic (Fig. 4D, F, G). The stomatal index is low (6.8–11 %) or medium (11–15.1 %). The tangential cell walls of subsidiary cells are even or curved.

Rarely, in the stomatal fields, there are bases of trichomae (Fig. 4D, E): large and round. Their area is about 1000–1100 μm^2.

Ptilophyllum. *P. caucasicum*. The leaves are pinnate, narrowly or broadly lanceolate, dissected into segments (Fig. 5A). The area of the segments ranges from 13.6 to 140 mm^2. Segments are alternate, widely spaced or closely pressed to each other. The shape varies from wide and short with a rounded top to narrow and long with a pointed apex.

Table 2. Statistical characteristics of the characters of the leaf structure for *Nilssoniopteris* and *Ptilophyllum*

Characters	*N. angustifolia*					*N. longifolia*				
	M	σ	m_x	X_{min}	X_{max}	M	σ	m_x	X_{min}	X_{max}
Ncell_upp	795	251	281	538	1234	797	255	266	444	1263
Supp	1466	421	518	952	2177	1373	524	458	648	2231
Per_upp	244	76	86	156	388	228	75	76	141	338
Sin_upp	1.58	0.30	0.56	1.25	2.08	1.54	0.29	0.51	1.08	1.93
Ncell_low	967	272	342	618	1453	759	214	253	516	1136
Slow	1326	210	469	1065	1632	1639	467	546	1030	2285
Per_low	215	40	76	156	271	270	94	90	156	450
Sin_low	1.47	0.21	0.52	1.16	1.72	1.65	0.38	0.55	1.10	2.45
Npap	524	133	185	303	744	0	0	0	0	0
Nstom	166	70	59	73	261	140	54	40	59	222
Lstom	37	8	9	32	39	31	10	10	30	48
Kstom	12.45	2.95	4.40	9.54	16.96	11.83	2.94	3.94	6.77	15.12
	P. caucasicum					*P. okribense f. ratchense*				
Ncell_upp	926	268	178	574	1773	803	113	207	535	953
Supp	2023	1234	389	607	4668	2284	1510	590	558	4830
Per_upp	341	140	66	152	646	380	138	98	199	635
Sin_upp	1.94	0.34	0.37	1.19	2.61	2.09	0.29	0.54	1.62	2.69
Ncell_low	1050	232	202	655	1629	938	168	242	561	1254
Slow	1870	771	360	301	3702	1837	1352	474	482	4392
Per_low	301	100	58	114	531	314	147	81	129	613
Sin_low	1.76	0.34	0.34	1.30	2.49	1.90	0.29	0.49	1.34	2.41
Npap	567	137	146	285	744	0	0	0	0	0
Nstom	111	38	21	56	179	156	60	40	59	255
Lstom	39	7	7	29	48	37	5	9	31	46
Kstom	8.86	3.05	1.70	4.35	13.98	12.11	2.79	3.13	7.07	16.88

Notes: M — mean; m_x — standard error of the mean; σ — standard deviation; X_{min} — minimum; X_{max} — maximum.

Characters: **Npap** — number of papillae per 1 mm^2 of epidermis. Abbreviations of the characters of epidermal structure are the same as in the Table 1.

The upper epidermis (Fig. 5B, Table 2) consists of small-sized cells (570–900 cells per 1 mm^2), medium-sized cells (900–1600 cells per 1 mm^2), or large-sized cells (1600–1770 cells per 1 mm^2). The projection of the area of the epidermal cells is square or rectangular, above the large veins it is rectangular. Outlines are sinuous-wavy, wide-wavy, sinuous. The coefficient of sinuosity of the tangential cell walls ranges from 1.2 to 2.6. Trichomae are absent.

The lower epidermis (Fig. 5C–F) consists of medium-sized cells (1350–1600 cells per 1 mm^2), or large-sized cells (655–1350 cells per 1 mm^2). The epidermis is divided into costal and stomatal fields (Fig. 5E). The projection of the area of the epidermal cells in the stomatal fields is rounded or square; in costal fields it is square, rectangular. Outlines are sinuous-wavy, wide-wavy, sinuous. The coefficient of sinuosity of the tangential cell walls ranges from 1.3 to 2.5. Most of the epidermal cells in the lower epidermis, both in costal and stomatal fields, have one central convex-shaped papilla. Their imprints are clearly visible on the upper side of the cuticle. On the lower side of the cuticle, they have narrow recesses-cavities in the central parts of the epidermal cells (Fig. 5C, D, E). The number of papillae varies from 285 to 745 per 1 mm^2. Trichomae are absent.

Leaves are hypostomatic. The stomata are arranged in two or three per width of stomatal field (Fig. 5E), their

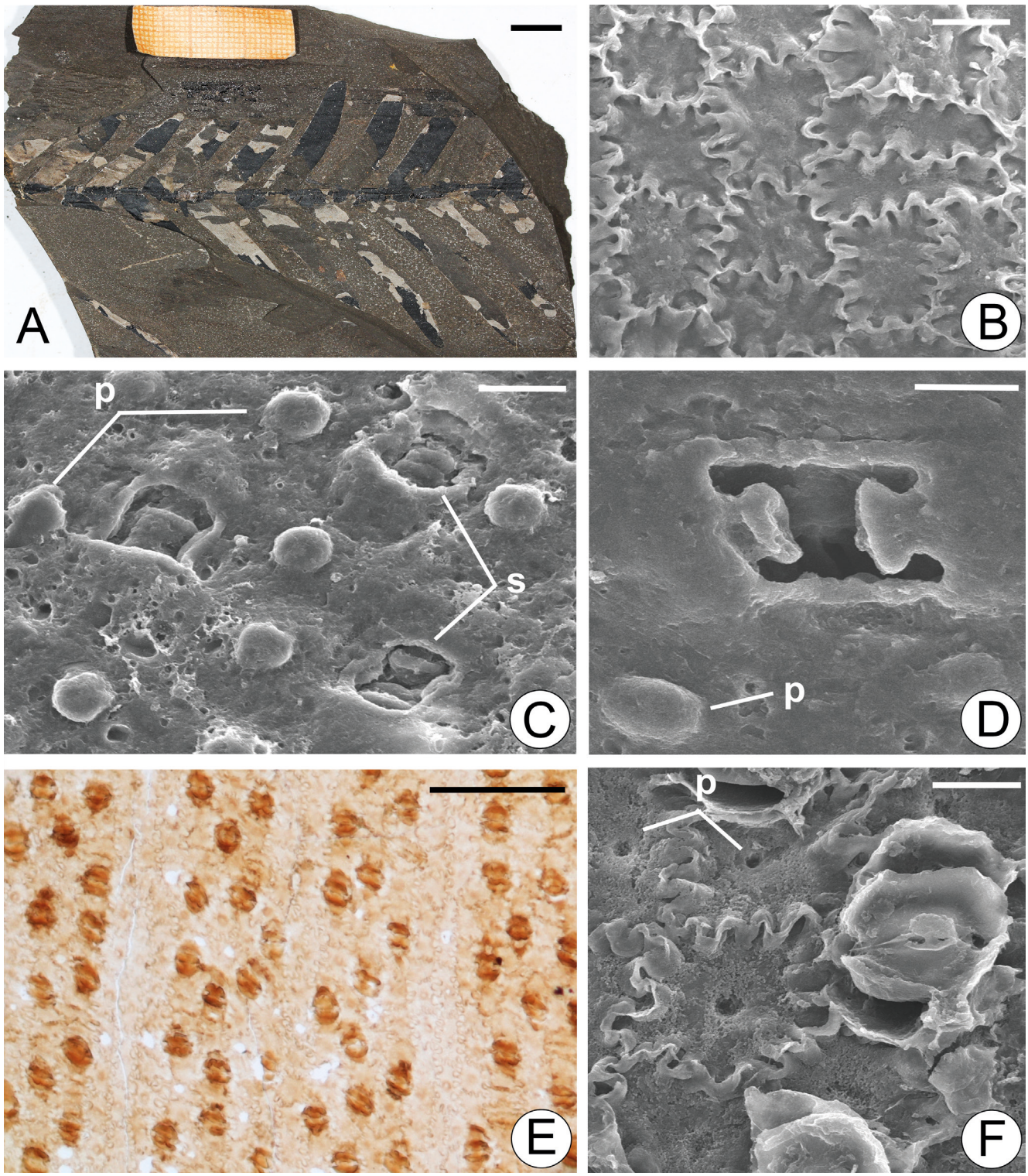

Fig. 5. Leaves of *P. caucasicum*. *A* — a fragment of leaf blade with segments, spec. BIN 1600; *B* — a cuticle fragment with imprint of upper epidermis, inner view, spec. BIN 1568; *C* — a cuticle fragment with imprint of lower epidermis (*s*) and papillae (*p*), external view, spec. BIN 1584; *D* — a stoma, external view, spec. BIN 1584; *E* — an abaxial side of leaf, a cuticle fragment with stomatal strips, spec. BIN 1635; *F* — a stoma and epidermal cells with imprints of papillae (*on*), inner view, spec. BIN 1607.

Scale bar: *A* — 1 cm; *B, D* — 20 μm; *C* — 50 μm; *E* — 200 μm.

apertures are oriented across the veins. Quite often, stomata are found in costal fields, less often they can be found above large veins. On the epidermal surface, there are very few (56–100 stomata per 1 mm^2), few (100–160 stomata per 1 mm^2), or average number of stomata (160–180 stomata per 1 mm^2). The type of stomata is paracytic (Fig. 5F). The stomatal index is very low (4.3–6 %), low (6–11 %) or medium (11–14.0 %). The stomata

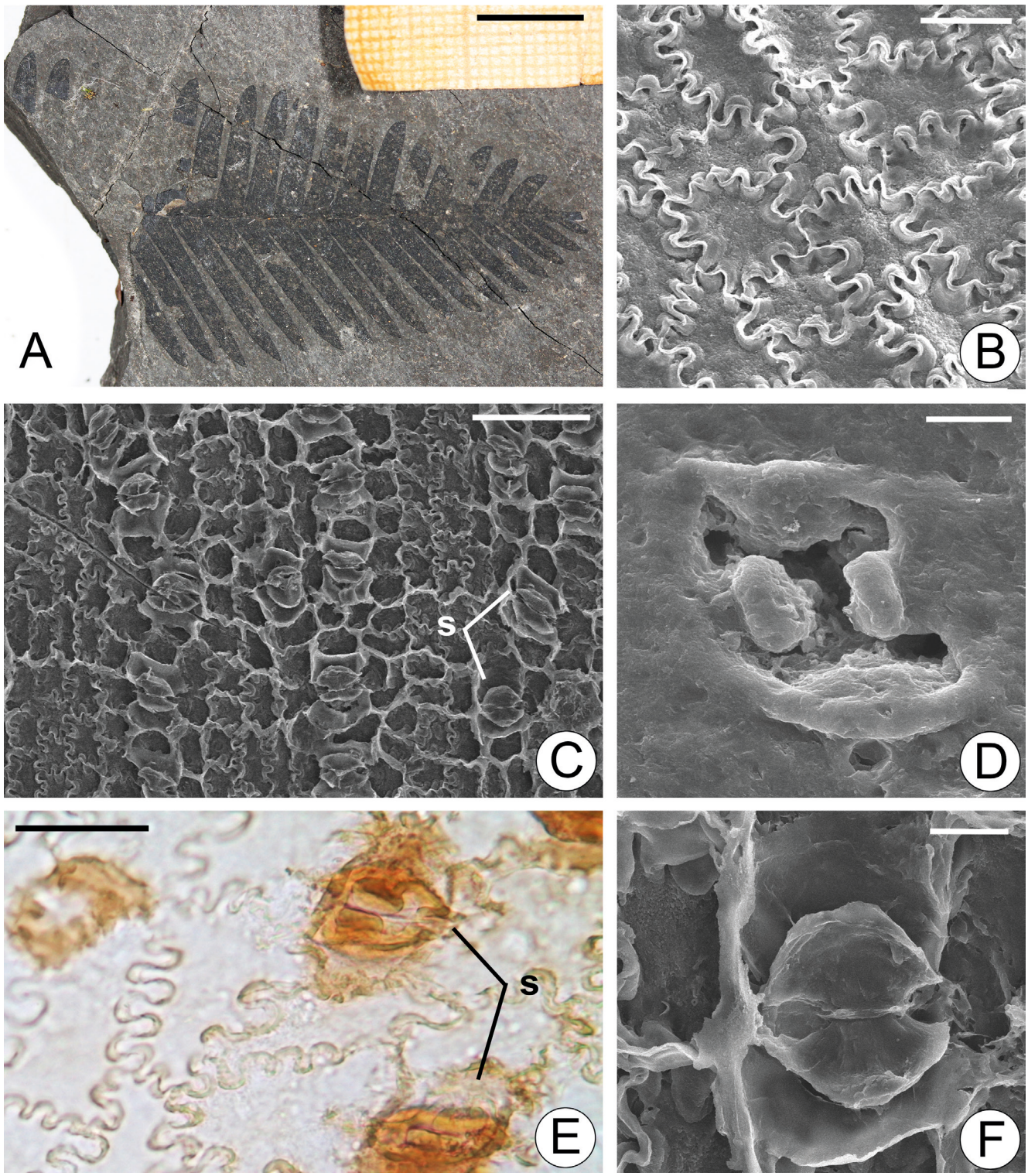

Fig. 6. Leaves of *P. okribense*. *A* — fragment of leaf blade with segments, spec. BIN 1573; *B* — cuticle fragment with imprint of upper epidermis, inner view, spec. BIN 1639; *C* — abaxial side of leaf, a cuticle fragment with stomatal strips; spec. BIN 1619; *D* — stoma, external view, spec. BIN 1573; *E* — abaxial side of leaf, a cuticle fragment with stomata (*s*), spec. BIN 1608; *F* — stoma, inner view, spec. BIN 1683.

Scale bar: *A* — 1 cm; *B* — 20 μm; *C, E* — 100 μm; *D, F* — 50 μm.

are submerged; the subsidiary cells hang over the guard cells, forming an H-shaped cavity (Fig. 5D).

P. okribense f. ratchense. The leaves are linear, dissected into segments (Fig. 6A). The segments are opposite or slightly offset from each other, narrow, long with a pointed apex. The area of the segments ranges from 8.4 to 270 mm^2.

The upper epidermis (Fig. 6B, Table 2) consists of medium-sized cells (900–950 cells per 1 mm^2), or large-sized cells (535–900 cells per 1 mm^2). The projection of

the area of the epidermal cells is square or rectangular; outlines are sinuous-wavy, wide-wavy, sinuous. The coefficient of sinuosity of the tangential cell walls ranges from 1.6 to 2.7. Trichomae are absent.

The lower epidermis (Fig. 6C–F) consists of large-sized cells (600–1253 cells per 1 mm^2), very large-sized cell (560–600 cells per 1 mm^2). The epidermis is divided into costal and stomatal fields (Fig. 6C). The projection of the area of the epidermal cells is flattened, square or rectangular, above the large veins it is rectangular. Outlines are sinuous-wavy and sinuous in costal fields, wide-wavy and sinuous-wavy in stomatal fields. The coefficient of sinuosity of the tangential cell walls ranges from 1.3 to 2.4. Trichomae are absent.

Leaves are hypostomatic. The stomata are located in indirect two-row stripes, their apertures are oriented across the veins (Fig. 6C). Stomatal fields may merge with each other. Stomata are often located in costal fields, less often — above large veins. On the epidermal surface, there are very few (59–100 stomata per 1 mm^2), few (100–160 stomata per 1 mm^2), or an average number of stomata (160–250 stomata per 1 mm^2). The type of stomata is paracytic (Fig. 6E, F). The stomatal index is low (7.1–11 %), medium (11–16 %) or large (16–16.9 %). The stomata are submerged; the subsidiary cells hang over the guard cells, forming an H-shaped cavity (Fig. 6D).

Table 3. Factor structure of epidermal characters in gnetums and Bennettitales

Characters	Factor 1	Factor 2	Factor 3
Ncell_upp	**785**	–231	478
Supp	**–831**	–256	151
Per_upp	**–889**	–271	232
Sin_upp	**–733**	–269	349
Ncell_low	**765**	–317	**527**
Slow	**–840**	–089	064
Per_low	**–896**	–169	184
Sin_low	**–740**	–244	280
Npap	–373	**–617**	–270
Nstom	**752**	**–565**	138
Ndstom	**643**	**–669**	–167
Lstom	**–600**	221	133
Kstom	–277	–444	**–784**
FD, %	52.5	14.3	12.1

Factors 1–3 — factor loadings on Factor 1 and Factor 2. FD — factor dispersion, %. Zero and the dot in front of the values of the characters are omitted. Characters with |r| > 0.5 are marked in bold. Abbreviations of the characters of epidermal structure are the same as in Table 1.

Correlations of the characters in epidermis of gnetums and Bennettitales. According to the results of PCA, three factors were revealed (Table 1). Their total factorial variance (∑FD) is 78.9 %.

Factor 1 (|r| ≥ 0.5; FD = 52.5 %) combined 11 main characters of epidermis: area of tangential cell walls of epidermal cells in upper and lower epidermis, their perimeter and sinuosity of the tangential cell walls, number of epidermal cells per 1 mm^2 of upper and lower epidermis, number of differentiated and aborted stomata per 1 mm^2 of epidermis, length of stomata. Character-indicator: perimeter of epidermal cells in lower epidermis (r = –0.896). The larger are epidermal cells, the larger are their area and perimeter, the lower is the sinuosity of the tangential cell walls, the lower is the number of stomata per 1 mm^2 of epidermis, but stomata are larger and most of them are functional. The lower are the epidermal cells, the lower are their area and perimeter, the higher the sinuosity of their tangential cell walls, the more stomata are per 1 mm^2, and the lower their size.

Factor 2 (|r| ≥ 0.5; FD = 14.3 %) included three characters of the epidermis: number of papillae per 1 mm^2, total number of stomata per 1 mm^2 and number of differentiated stomata per 1 mm^2. Character-indicator: number of differentiated stomata per 1 mm^2 of epidermis (r = –0.669). This factor shows differences in epidermal structure among Bennettitales, which have central papillae on the surface of the epidermal cells (*N. angustifolia* and *P. caucasicum*), Bennettitales without papillae (*N. longifolia* and *P. okribense f. ratchense*) and gnetums. For Bennettitales with papillae: the more stomata are per 1 mm^2 of epidermis, the more papillae are in the epidermis. The fewer the number of stomata per 1 mm^2 of epidermis, the fewer number of papillae are in the epidermis. For gnetums: the higher the total number of stomata per 1 mm^2 of epidermis, the more of them are differentiated. The lower the total number of stomata per 1 mm^2 of epidermis, the fewer of them are differentiated.

Factor 3 (|r| ≥ 0.5; FD = 12.1 %) includes two characters: stomatal index and number of epidermal cells per 1 mm^2 of lower epidermis. Character-indicator: stomatal index (r = –0.784). This factor describes the ratio of the specific proportion of stomata and the size of epidermal cells. The larger are epidermal cells, the lower is the stomatal index. The smaller are epidermal cells, the higher is the stomatal index.

In the diagram of factor space (Fig. 7), the specimens of the investigated leaves diverge along the factor 1. Gnetums form a fairly dense cluster, in which individual species stand out well. On the other hand, Bennettitales form a large cluster that occupies a significant part of the factor space. It is rather problematic to single out individual species or genera within this cluster. Nevertheless, the second factor more or less distinguishes two clusters, including species with central papillae on the epidermal cells (*N. an-*

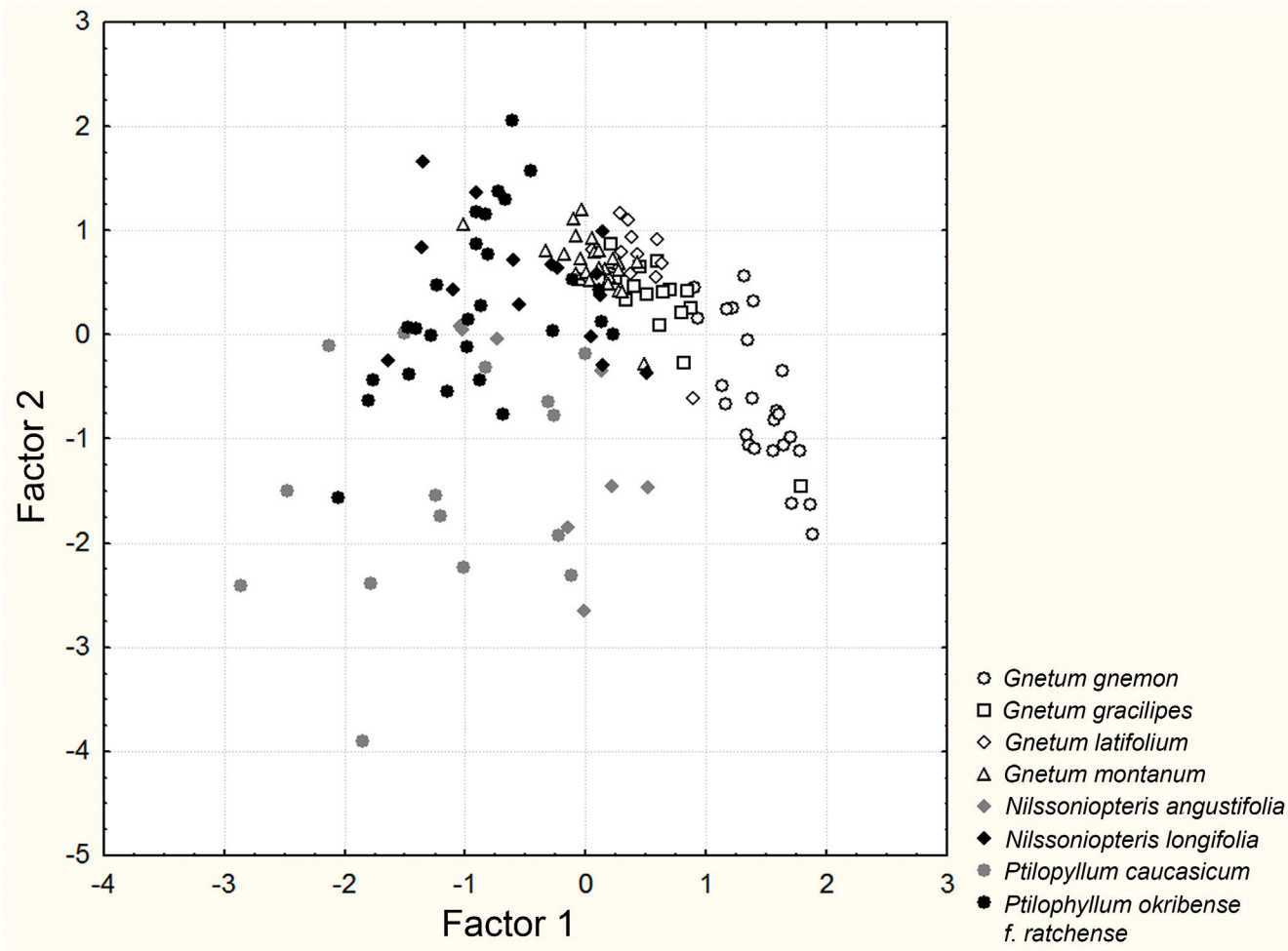

Fig. 7. Distribution of leaf samples of gnetums and Bennettitales in the scatterplot of Factor 1 and Factor 2.

Gnetum species are marked with white outline icons; Bennettitales with papillae in epidermal cells are marked by gray icons; Bennettitales species without papillae are marked by black icons.

gustifolia and *P. caucasicum*) and species without papillae (*N. longifolia* and *P. okribense f. ratchense*).

As already shown in previous articles, the epidermis of gnetums is rather similar. Differences between species were revealed in the ratio of the structural elements of epidermis, as well as in the number of aborted stomata and their ratio with the number of differentiated stomata (Pautov, Pagoda, and Krylova, 2012; Pautov and Pagoda, 2015). Aborted stomata are found in the epidermis of many species of *Gnetum*. Using *G. gnemon* as model species, it has been shown that their origin occurs during the second phase of leaf development during the period of mass differentiation of paracytic stomata (Pautov and Pagoda, 2013, 2014). The ratio of differentiated and aborted stomata is reflected in the correlation structure of a leaf (Pautov, Pagoda, and Krylova, 2012). Presumably, the presence of aborted stomata may be a kind of mutation developed in the course of evolution for effective regulation of transpiration.

Epidermis of Bennettitales is very diverse even within the same species. Nevertheless, despite strong differences in the epidermal structure, a number of similarities were revealed in the correlation structure of epidermis of gnetums and Bennettitales. Firstly, the similarity in the ratio of the elements that form the epidermis (Factor 1, Table 3): number of epidermal cells and number of stomata per 1 mm^2 of epidermis. The revealed correlations in this case are the basic characteristics of the epidermis and are also inherent in flowering plants (Pautov, 2009). Secondly, the similarity of correlations between the size of the leaf (its large-sized and small-sized cells) and the degree of sinuosity of tangential cell walls of the epidermal cells (Factor 1, Table 3). The shape of the epidermal cells plays a significant role in the leaf morphogenesis of seed plants (Pautov, 2009; Pautov and Vasilieva, 2010). This character can, in particular, be involved in maintaining the flat shape of the leaf blade, and affect the density of stomata. Thirdly, in gnetums and Bennettitales, a negative correlation was found between the size of stomata and the size of epidermal cells (Factor 1, Table 3). This can be explained as an ontogenetic character of leaf development (the more cells have divided, the more small-sized they are, and

epidermis contains many small stomata; the fewer cells have divided, the more large-sized they are, and epidermis contains a low number of large stomata), as well as an increase in the efficiency of transpiration regulation.

Character variability in the epidermis of gnetums and Bennettitales. The coefficient of variation of the investigated characters in epidermis of *Gnetum* ranges from 6.4 to 24.0 %. Most of them have medium and high levels of common variability. A very low level (Cv ≤ 7.5 %) is for the length of stomata. Low level (8–12 %) is for sinuosity of epidermal cells in upper and lower epidermis. Medium level (13–20 %) is for stomatal index, the perimeter of epidermal cells in upper and lower epidermis and the number of epidermal cells per 1 mm^2 of lower epidermis. Increased level (21–30 %) is for the area of tangential cell walls of epidermal cells in upper and lower epidermis, the number of epidermal cells per 1 mm^2 of upper epidermis and the number of stomata per 1 mm^2 of epidermis.

The coefficient of variation of the investigated characters in the epidermis of *Nilssoniopteris* ranges from 18.0 to 39.9 %. Most of them demonstrate increased and high levels of common variability. The medium level of variability is for sinuosity of epidermal cells in upper and lower epidermis. Increased level is for stomatal index, the perimeter of epidermal cells in lower epidermis, the number of epidermal cells per 1 mm^2 of lower epidermis. High level (31–40 %) is for the length of stomata, the area of tangential cell walls of epidermal cells in upper epidermis, perimeter of epidermal cells in upper epidermis, the number of epidermal cells per 1 mm^2 of upper epidermis, the number of stomata per 1 mm^2 of epidermis.

The coefficient of variation of the investigated characters in the epidermis of *Ptilophyllum* ranges from 15.7 to 63.5 %. Most of them have increased and high levels of common variability. The average level of variability is for sinuosity of epidermal cells in upper and lower epidermis, the length of stomata, the number of epidermal cells per 1 mm^2 of lower epidermis Increased level is for the number of epidermal cells per 1 mm^2 of upper epidermis, stomatal index. High level is for the number of stomata per 1 mm^2 of epidermis, the perimeter of epidermal cells in upper and lower epidermis. Very high level (Cv > 40 %) is for the area of tangential cell walls of epidermal cells in the upper and lower epidermis.

In the diagram in Fig. 8, the values of the coefficients of variation are shown. The characters of epider-

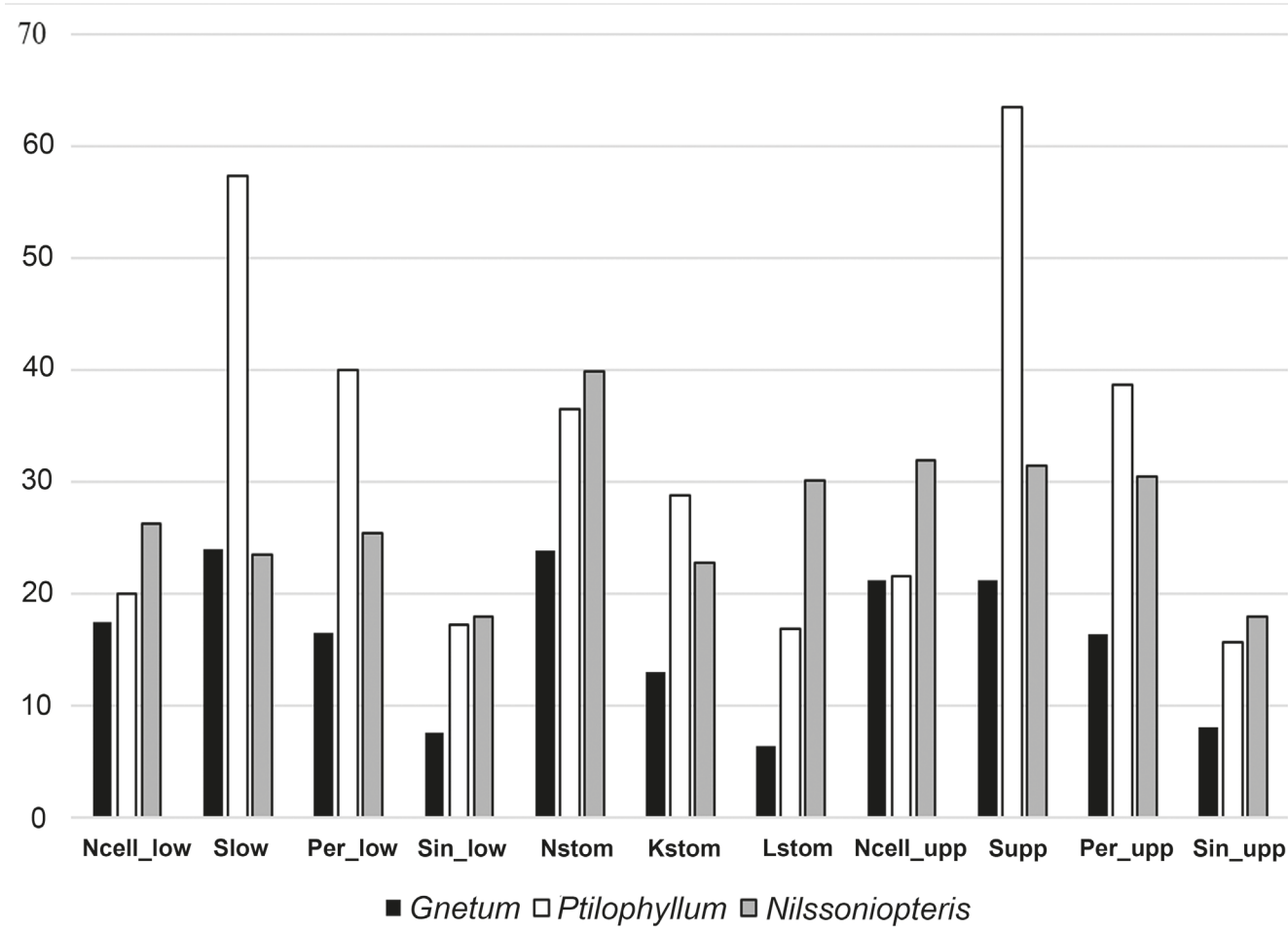

Fig. 8. The common variability of the leaf structure characters in gnetums and Bennettitales.
The vertical axis indicates the coefficient of variation (Cv, %). Abbreviations of the characters of epidermal structure are the same as in Table 1.

mal structure in Bennettitales are generally higher than those of gnetums. The common variability of characters in *Nilssoniopteris* and *Ptilophyllum* remains at increased and high levels, while in gnetums they have medium and increased levels. Nevertheless, in general, the values of the coefficients of variation in characters of Bennettitales and gnetums are comparable and, despite a clear scatter in values, they demonstrate a number of similarities.

Firstly, in both gnetums and Bennettitales, the sinuosity of the tangential cell walls of the epidermal cells, compared with the variability in other characters, has the lowest level of variability (Cv ≤ 18.0 %), i. e., this is a fairly stable character. This is consistent with taxonomic concepts. The sinuosity of the tangential cell walls in epidermal cells is successfully used as a diagnostic character for the determination of Bennettitales species (Doludenko and Svanidze, 1969; Kiritchkova and Nosova, 2012; Rudall and Bateman, 2019). Secondly, the length of stomata is also a stable character. It is relatively invariable in species of *Ptilophyllum* (Cv = 16.8 %) and slightly variable in gnetums (Cv = 6.4 %). The size of the stomata also has a taxonomic significance for *Ptilophyllum* (Doludenko and Svanidze, 1969). Thirdly, there is considerable variation in the values of the coefficients of variations for the number of epidermal cells per 1 mm^2 of upper and lower epidermis (17.5 % ≤ Cv ≤ 31.9 %), the area of tangential cell walls of epidermal cells in upper and lower epidermis (21.2 % ≤ Cv ≤ 63.5 %), the number of stomata per 1 mm^2 of epidermis (29.3 % ≤ Cv ≤ 39.9 %). These characters determine the size of epidermal cells and, consequently, the leaf size, which is consistent with the results of PCA (Factor 1, Table 3). Nevertheless, despite significant difference in the level of common variability of the structural elements of epidermis in Bennettitales, the ratio in the number of stomata and the number of epidermal cells — the stomatal index (13.0 % ≤ Cv ≤ 28.8 %) — remain more or less stable not only within the species, but within the genera as well.

Conclusion

The investigated structural organization of the epidermis of the leaves of Bennettitales is comparable and similar in many aspects to the leaves of extant gnetums. Given that both groups are considered as possible ancestors or closer relatives of flowering plants (Crane, 1996; Friis et al., 2007; Sokoloff and Timonin, 2007; Gambaryan and Kuznetsov, 2021), it will be further possible to compare the obtained results with those on primitive flowering plants. Perhaps it could be possible to reveal some general directions of leaf evolution based on the transformation of quantitative characters.

Leaves of Bennettitales and especially their stomatal characters have become central to their description, identification and systematization. Although these characters are also open to considerable interpretation and debate. Rudall and Bateman (Rudall and Bateman, 2019) identify close similarities between paracytic stomata of Bennettitales and relict species *Gnetum*. They indicate that the pair of lateral subsidiary cells of Bennettitales, as in *Gnetum*, are both mesogene cells. We found significant similarities in the variability and correlation structure of the epidermal structure of the extinct Bennettitales *Nilssoniopteris* and *Ptilophyllum* and extant gnetums.

An important question is whether the similarities in leaf morphology between Bennettitales, *Gnetum* and flowering are related to their shared ancestry or indicate shared ecological adaptations.

Rudall and Bateman (Rudall and Bateman, 2019) concluded that stomata of *Gnetum* represent by far the most likely analogue for the stomatal development in Bennettitales. The similarities in correlational structure of Bennettitales and gnetums could also represent an ecophysiological marker for adaptation or developmental features because many correlations in structural organization are basic for seed plants, especially the correlation between the size of the leaf and the degree of sinuosity of tangential cell walls of the epidermal cells, which indicate the shape of the epidermal cells. Correlation between the size of stomata and size of epidermal cells could be explained both as ontogenetic character of leaf development and as regulation of transpiration.

The present study provides the first successful attempt to evaluate the structural organization of the epidermis in leaves of Bennettitales and compare it with the leaves of extant plants using a principal component analysis. Together, these features could infer the development to this diverse and relatively derived lineage that co-existed with the earliest recognizable angiosperms, and help to use these characters in phylogeny reconstructions. However, the data obtained only on the cuticle of fossil plants are not enough to get a complete picture.

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