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Development of the sphagnoid areolation pattern in leaves of Palaeozoic protosphagnalean mosses

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- **Background and Aims** Protosphagnalean mosses constitute the largest group of extinct mosses of still uncertain affinity. Having the general morphology of the Bryopsida, some have leaves with an areolation pattern characteristic of modern *Sphagna*. This study describes the structure and variation of these patterns in protosphagnalean mosses and provides a comparison with those of modern *Sphagna*.
- **Methods** Preparations of fossil mosses showing preserved leaf cell structure were obtained by dissolving rock, photographed, and the resulting images were transformed to graphical format and analysed with Areoana computer software.
- **Key Results** The sphagnoid areolation pattern is identical in its basic structure for both modern *Sphagnum* and Palaeozoic protosphagnalean mosses. However, in the former group the pattern develops through unequal oblique cell divisions, while in the latter the same pattern is a result of equal cell divisions taking place in a specific order with subsequent uneven cell growth. The protosphagnalean pathway leads to considerable variability in leaf structure.
- **Conclusions** Protosphagnalean mosses had a unique ability to switch the development of leaf areolation between a pathway unique to *Sphagnum* and another one common to all other mosses. This developmental polyvariancy hinders attempts to classify these mosses, as characters previously considered to be of generic significance can be shown to co-occur in one individual leaf. New understanding of the ontogeny has allowed us to re-evaluate the systematic significance of such diagnostic characters in these Palaeozoic plants, showing that their similarity to *Sphagnum* is less substantial.

Key words: Cell divisions, leaf areolation, cell packets, leaf morphogenesis, fossil, Permian, Palaeozoic, Protosphagnales, *Vorcutannularia*, *Intia*, *Kosjunia*, *Junjagia*, *Sphagnum*.

INTRODUCTION

Reconstruction of cell division sequences by means of modelling based on anatomical studies remains a rather poorly explored field in botany. Progress in this direction has been achieved only in a few instances. One of these was the recurrent patterns of cell sequences in radially organized organs of vascular plants studied by Peter Barlow (Barlow, 1982; Lück *et al.*, 1994, 1997; Barlow *et al.*, 2001; Barlow and Lück, 2004*a*, *b*, 2006). Broad parallels can be drawn from these studies, including heterocyst formation in the cyanobacterium *Anabaena*, similar in its idio-blast formation through unequal cell divisions in the final stages of differentiation of cell packets, i.e. a group of cells derived from a single mother cell (Barlow and Lück, 2008).

Moss leaves provide a convenient model for studying cell packets, as the leaf lamina is usually unistratose and each cell packet in a moss leaf develops from a single cell that normally divides 8–14 times (4–7 to the left, 4–7 to the right). Leaf areolation is often patterned, showing an outline of cell packets (Lorenz, 1864; Pottier, 1925; Frey, 1970; Bopp, 1984), which are especially apparent in small leaves. Cell packets of 16 (4 × 4), 32 (8 × 4) to

64 (8 × 8) cells in moss leaves can usually be unequivocally interpreted in terms of sequences of cell division (Donskov, 2015). In contrast, for large moss leaves consisting of thousands of cells, such interpretations are difficult or almost impossible to make. This is due primarily to stochastic cell displacements and unequal elongation as a result of pressure from neighbouring cells during later stages of differentiation. There are only a few exceptions which can provide opportunities to study the late stages of leaf lamina development through specific areolation patterns, and one of these are protosphagnalean mosses.

Mosses of the order Protosphagnales have been shown to be widespread in the late Palaeozoic, especially during the Permian period in Angaraland and the part of Subangaraland (Neuburg, 1960; Ignatov, 1990). They are abundant in these deposits and their fossils can be extracted using the bulk macer-ation method (Darrah, 1960; Andrews, 1961). Cellular structure is excellently preserved in these Palaeozoic plants and it can be used to reconstruct the sequences of cell divisions, because cell packets can be observed in many cases as clearly as in modern mosses of the genus *Sphagnum*.

Certain details of protosphagnalean cell areolation are similar to those of modern Sphagnopsida, one of the most basal lineages in the evolution of mosses (Newton *et al.*, 2007). Neuburg (1960) and Ivanov *et al.* (2015) presume that sphagnalean mosses originated from protosphagnalean ancestors, while Abramov and Savicz-Ljubitskaya (1963) thought that the genus *Intia* Neuburg [a protosphagnalean genus according to Ignatov (1990) and Ivanov *et al.* (2015)] is simply a member of the extant family Mniaceae. Protosphagnalean mosses are, indeed, similar to that family and differ from *Sphagna* in growth habit and their sparsely arranged broad leaves with a single costa. However, details of their lamina areolation may approach those of *Sphagnum*, whose relationship to protosphagnalean mosses remains an intriguing topic, which becomes more complicated due to an extraordinary diversity of the extinct mosses.

Areolation in *Sphagnum* was described by Schimper (1858), and the most detailed study was published by Schnepf (1973). It can be readily recognized by having a network of alternating large inflated hyalocysts and much narrower chlorocysts, surrounding each hyalocyst. This pattern is a result of unequal and oblique cell divisions initiated at a very early stage of development (Fig. 1S1). An important characteristic of *Sphagnum* areolation is the strong elongation of the 'first chlorocyst cell'

at its distal end, while the 'second chlorocyst cell' remains less elongated and joined to the 'first chlorocyst cell' in its middle part (marked as α and β in Fig. 1S1′, S2′ and S3′). Such an arrangement of three cells, one octagonal and two pentagonal, we hereafter refer to as the 'Sphagnoid Areolation Pattern', or SAP. For the discussion below, the SAP cells are numbered and referred to as 'SAP cell 1' (octagonal cell, equivalent to the first chlorocyst), 'SAP cell 2' (equivalent to the second chlorocyst), 'SAP cell 3' (equivalent to hyalocyst in *Sphagnum*) (cf. Figs. 1, 2 and 3). SAP also includes variants in which some of the cells of its basic structure undergo subsequent divisions. Protosphagnalean mosses have one or two additional divisions, resulting in 'SAP cell 4' and 'SAP cell 5' (Fig. 2PD).

Given recent findings of *Sphagnum*-like remains in upper Ordovician deposits (Cardona-Correa *et al.*, 2016), which is consistent with molecular phylochronological dating (Newton *et al.*, 2007), the opposite direction of evolution, from Sphagnales to Protosphagnales, is not impossible, although the lack of data precludes a wider discussion of this problem. Regardless of this, our data on the two types of laminal cell areolation clarifies the specifics of leaf growth in these large moss lineages. Thus, in the present study we focus on structural, and potentially also developmental, differences between sphagnalean and protosphagnalean mosses.
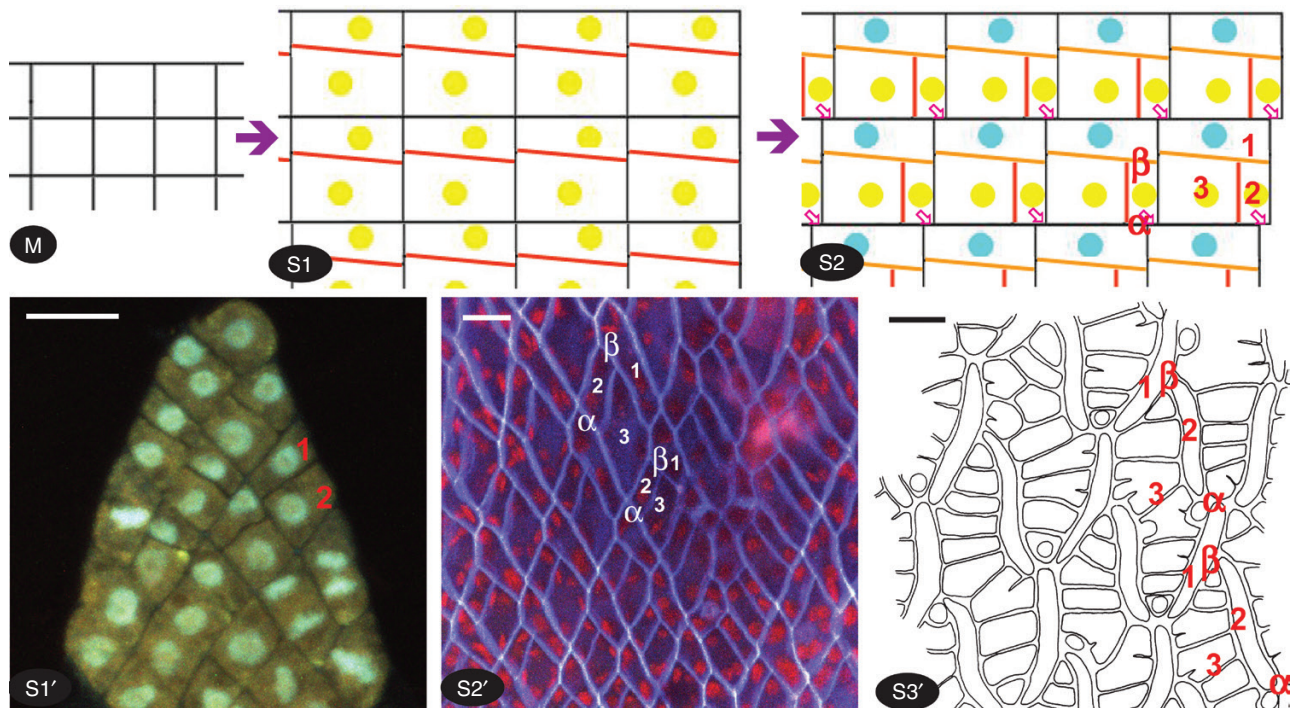

FIG. 1. Leaf areolation development in *Sphagnum*. (M) Meristematic stage, where cells are quadrate as a result of rapid division without displacements. (S) *Sphagnum* areolation pattern development. Schemes S1 and S2 show first and second divisions, respectively, in mother cells of cell triads that form a *Sphagnum* leaf. Scheme S2 also illustrates the displacement due to cell wall growth in parts indicated by open pink arrows. Micrographs S1′ and S2′ (*Sphagnum girgensohnii* Russ., extant living plants from Moscow area, young stem leaves) illustrate stages S1 and S2, and drawing S3′ shows developed *Sphagnum* areolation in a stem leaf of *S. tenellum* (Tver Oblast, 22 September 1931 Tyuremnov, s.n. MW). (S1′) Autofluorescence + DAPI staining in light blue of nuclei and chromosome position in mitosis; (S2′) autofluorescence, showing cell walls (blue) and chloroplasts (red). Cell colours in schemes correspond to the number of corners in cells: four, deep blue; five, yellow; six, red; seven, green; eight, light blue. Note that 'corner' means here the junction point of three cells. The sphagnoid areolation pattern, SAP, appears after two divisions of the SAP mother cell, forming a triad of cells with eight, five and five corners. In the selected picture, SAP cell numbers are shown, where '1' is the octagonal 'first chlorocyst', the smaller cell from the first division of the 'SAP mother cell', '2' is the pentagonal 'second chlorocyst' and '3' is the pentagonal cell further developing into 'hyalocyst'. The ends of SAP cells 2 are conspicuously different: the end marked by α connects the joint of octagonal cells of the neighbouring row, while the end indicated by β is attached to the middle part of SAP cell 1. Scale bars for S1′ and S2′ = 10 μm; S3′ = 20 μm.
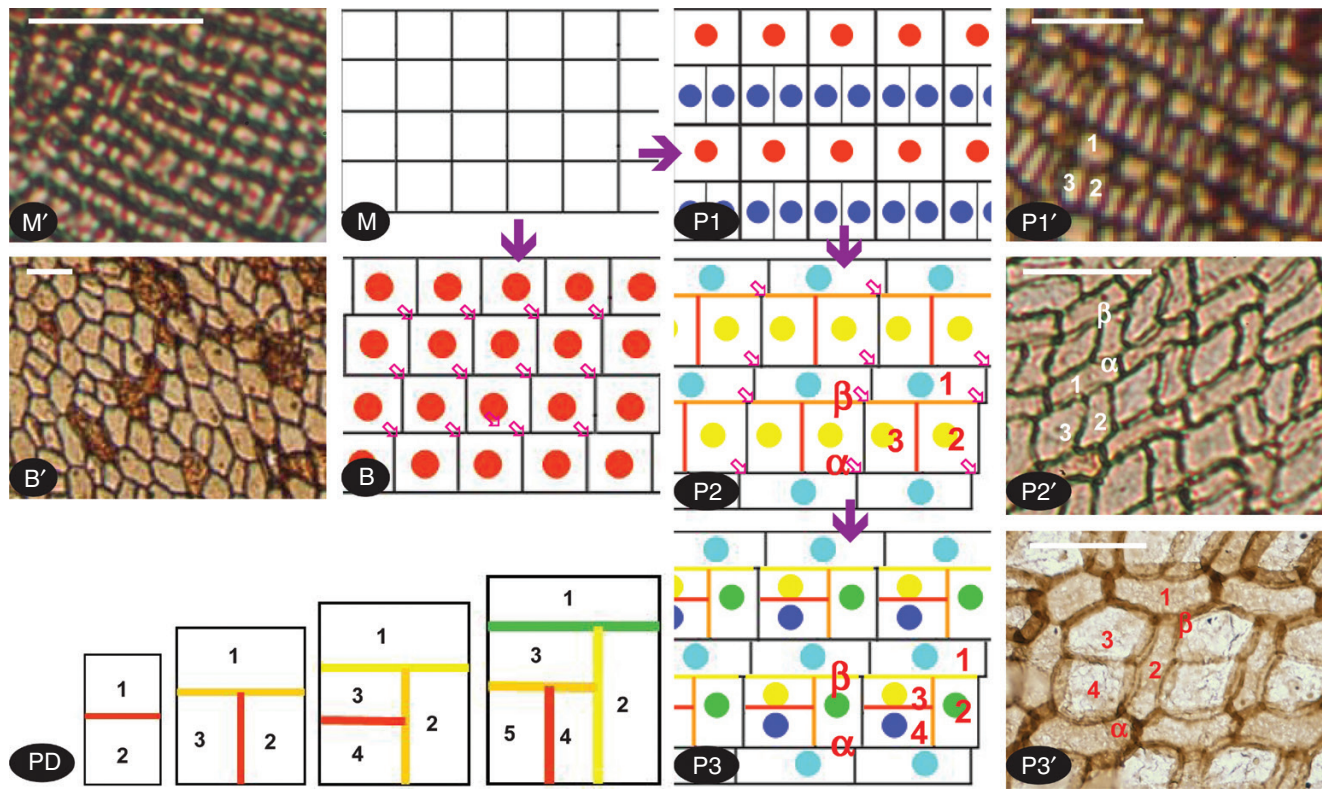
FIG. 2. Leaf areolation development in Protosphagnales. (M) Meristematic stage, where cells are quadrate as a result of rapid divisions without displacements, scheme (M) and example (M′, close-up from Fig. 3I). (B) Bryoid areolation of cells with six corners, formed from quadrate cells as a result of displacements indicated by open pink arrows; initially almost quadrate in shape, they later develop into hexagonal cells, which is most common in most modern mosses, class Bryidae, and occasionally occurring in some protosphagnaleans, shown for example in B′, a close-up from the leaf in Fig. 6. (P) Protosphagnoid areolation development. (P1) A pattern common for protosphagnalean mosses where distinctive rows of larger cells alternate with rows of doubled number of cells, originally without displacement. (P2) The result of displacement from the pattern shown in P1, the 8 + 5+5 pattern (triads of one octagonal and two pentagonal cells). (P3) The next stage, the 8 + 7+5 + 4 pattern (tetrads with eight, seven, five and four corner cells), appearing from one more division in P2. (P1′–P3′) Micrographs (from specimens shown also in Fig 3M, H, J) corresponding to schemes in P1–P3. (PD) Scheme of cell divisions forming SAP in protosphagnalean mosses: note that the last stage in this series may be interpreted as a result of asymmetric cell divisions, although observations on cell variation in fossils indicate that cell divisions are largely equal, but followed by unequal cell wall growth. Cell division order is indicated by different colours, the most recent division being red, and then orange, yellow and green. For colour labelling of the number of corners see legend to Fig. 1. Scale bars for all micrographs 50 μm.

MATERIALS AND METHODS

Collections

Material was collected in the Pechora Coal Basin (north-eastern European Russia, approx. 150 km west of Vorkuta). The deposits have been dated to the Kazanian Stage of the Upper Permian period (260–270 Mya); for more details see Maslova *et al.* (2012*b*). Numerous fragments of various protosphagnalean mosses were extracted by bulk maceration of the rock matrix in hydrofluoric acid. The moss material was then mounted in glycerol–gelatin slides, and photographed under a compound microscope with a digital camera. About 3000 samples comprising almost more or less entire leaves and leaf fragments were studied (Maslova *et al.*, 2012a,b; Maslova and Ignatov, 2013). For the narrower scope of the present study, we focused particularly on specimens showing rapidly dividing small-celled areas.

Cells in these areas are close to the lower limit of possible cell size in mosses, near 5 μm. Maslova *et al.* (2012b) showed that such cells may occur not only in the basal portion of leaves, as common in modern mosses, but also in upper or middle leaf portions. About 50 leaf fragments representing different morphotypes were included in the detailed analysis. The morphotypes discussed here are attributed to the genera *Intia*, *Junjagia* Neuburg, *Protosphagnum* Neuburg, and *Vorcutannularia* Pogor. ex Neuburg as described by Neuburg (1960), and *Kosjunia* Fefil. as described by Fefilova (1978). Most of them include areas where the SAP arrangement is shown.

Areoana analysis

Photographs of mostly undamaged leaf fragments were digitized using the custom designed Areoana software (Ivanov and Ignatov, 2011, 2013). Cell walls were manually outlined on digital micrographs (Fig. 4D), and these images were transformed into a graphical format in the same program (Fig. 4B). To construct the cell network, Areoana connects the points of junction of three or four cells by straight lines, thus representing the cells as polygons. This approximation provides a number of quantitative characteristics for each cell, of which this paper presents data on area, number of corners (=number of sides),
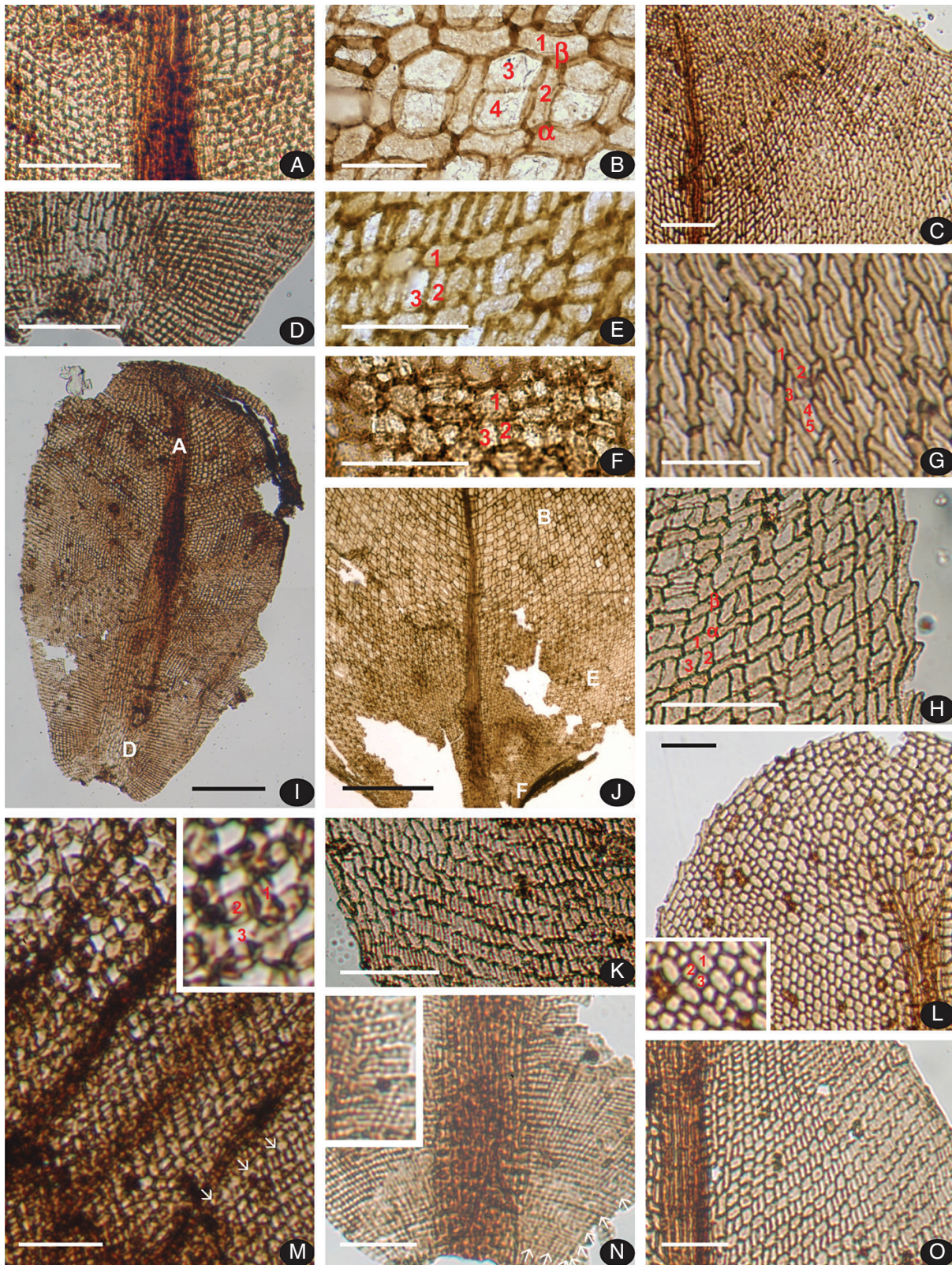
Fig. 3. Protosphagnalean mosses, demonstrating variation of areolation patterns in leaves and its parts. (A, D, I, M) *Junjagia*; (B, E, F, J) *Vorcutannularia*; (C, G) *Protosphagnum nervatum* Neuburg; (H, K, N) *Intia variabilis* Neuburg; (L, O) *Kosjunia*. In selected pictures SAP cells numbers are shown and specific ends of SAP cells 2 are marked by α (end to joint of octagonal cells of the neighbouring row), and β (end of SAP cell 2 attached to middle part of SAP cell 1). Position of A and D are shown in I and those of B, E and F in J. Specimens: A, D, I: 32M_4_18_2, B, E, F, J: 32M_5_5_1; C: 32M_2_28_3; G: A-41-3_100_1; H: 32M_5_15_1; K: 2M_6_45_1; L, O: 32M_7_1_1; M: 32M_4_24_1; N: 2M_9_24_1. Scale bars: A, C, D, G, H, K–O = 100 μm; B, E, F = 50 μm; H, I = 200 μm; J = 0.5 mm.
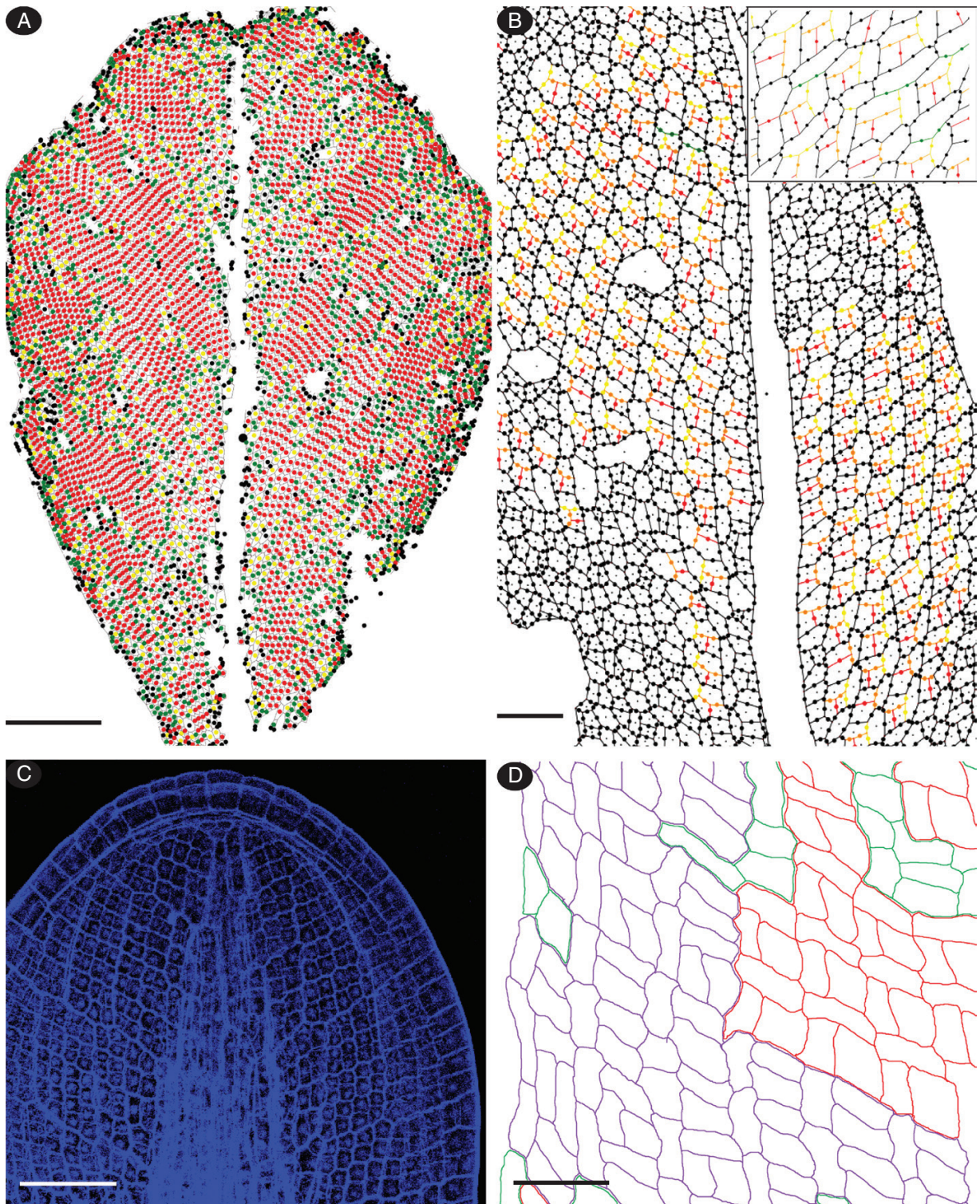
FIG. 4. (A) Digitized image of a mature leaf of *Rhizomnium tuomikoskii* (specimen: Ignatov 06_2997 MHA); images for A were originally obtained with a polarizing microscope, and then digitized and processed in Areoana, contrasting cell packets 8 × 8 (cf. C), as areas where hexagonal cells predominate. (B) SAP in a protosphagnalean moss *Vorcutannularia*, 32M_9_15, represented in a digitized image with cell walls marked according to the order of cell divisions as shown in Fig. 2PD. (C) Juvenile leaf of *R. tuomikoskii* (same specimen as in A): cell walls stained with fluorescent brightener, showing aligned cell packets 2 × 2 and already somewhat offset subquadrate cell packets 4 × 4. (D) Manually outlined areolation of *Intia variabilis* (same specimen as in Fig. 3H), showing changes of cell row orientations from transverse (red) to longitudinal (pink),with uncertain cases marked in green; similar patterns are seen in B. Scale bars: A = 0.5 mm; B = 100 µm; C, D = 50 µm. For colour legends see Fig. 1 (for number of corners) and Fig. 2 (for cell division order).
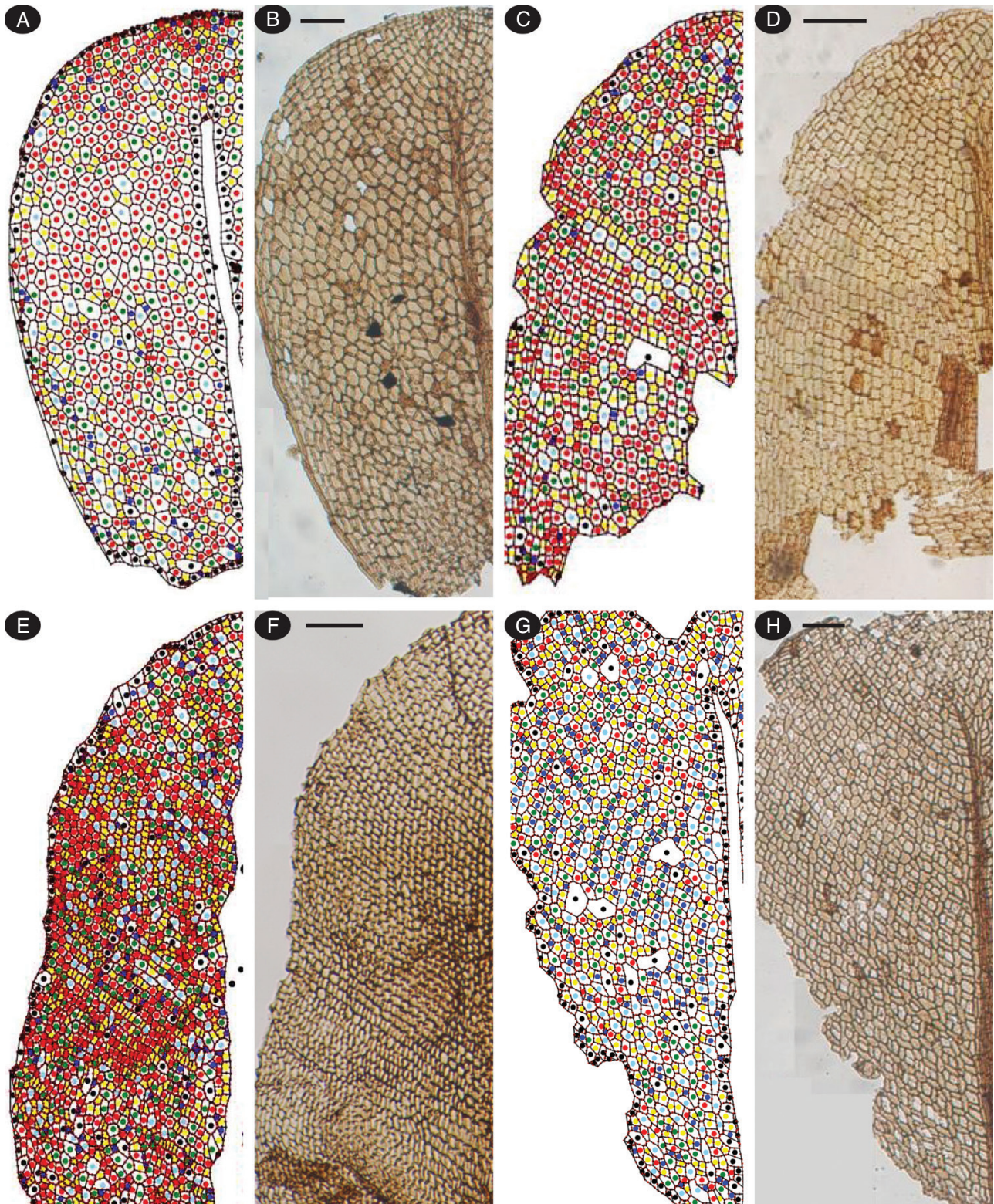
Fɪɢ. 5. Pairs of digitized images and micrographs, showing progressive increase of SAP occurrence in leaves of different protosphagnalean species. (A, B) Young leaf, *Intia* sp., 32M_2_16_1. (C, D) Moderately young leaf, *Intia* cf. *variabilis*, 32M_2_16_1. (E, F) Developed leaf, *Intia* cf. *variabilis*, 32M_14_9_5. (G, H) Developed leaf, *Vorcutannularia* sp., 32M_9_15. SAP is moderately developed in A, C and E, where it appears in yellow+light blue assemblages, either scattered (A) or linear (C), among Bryoid areolation patterns, where red dots prevail. G represents a more strongly developed SAP, where assemblages of cells with 8 + 7+5 + 4 corners (dark blue+green+light blue+yellow) almost totally substitute the Bryoid pattern. Scale bars: A = 50 µm; B and D–M = 100 µm; C = 0.5 mm. For colour legend see Fig. 1.

length and width. A useful option is to mark in different colours cells with different numbers of corners. This provides a better visual contrast of the SAP distribution within the leaf, as SAP is formed most commonly by assemblages of either one octagonal and two pentagonal cells (8 + 5+5 cells) or 8 + 7+5 + 4 cells, i.e. totally lacking hexagonal cells, which are the main constituent in modern moss leaves (Fig. 4A, C) and also occur in some protosphagnalean mosses as well (Figs 2B′, 5 and 6).

Another option available in Areoana is reconstruction of the sequence of cell divisions. The order of cell divisions that lead to formation of the SAP (Fig. 2P2, P3) can only be that shown in Fig. 2PD. Coding cell divisions in colours according to their order as shown in Fig. 2PD (cf. also Fig. 4B) allows us to reconstruct their sequence up to the last two divisions (in tetrads), last three divisions (in tetrads) and last four divisions (in pentads). This makes it possible not only to visualize the sequence of the latest divisions, but also to compare parameters for the SAP cell 1, SAP cell 2, etc. (as numbered on the diagram in Fig. 2PD). The parameters compared are surface area and length to width ratio, and were measured for a total of 8976 cells in 2194 SAP units from six leaves, including a large *Vorcutannularia* leaf (Fig. 6H) that was measured separately for distal and proximal parts (Table 1). It is notable that the SAP pattern observed in fully developed leaves (Fig. 2PD) appears to be the result of a series of unequal cell divisions. However, these divisions can in fact probably be equal, while subsequent changes in cell shape and size, due to cell growth in the course of development, modify them to the shapes seen in mature leaves, i.e. as in Fig. 2PD.

Along with the fossil mosses, the study also includes two extant mosses, *Sphagnum* L. and *Rhizomnium* (Mitt. ex Broth.) T.J. Kop., selected for comparisons of cell type distribution. Young leaves of *Sphagnum girgensohnii* Russow were photographed under an Olympus FV-1000 laser confocal scanning microscope (LSCM), using violet and blue lasers λ=405 + 473 nm, with 4′,6-diamidino-2-phenylindole (DAPI) and berberin staining, without exclusion of autofluorescence (Fig. 1S1′, S2′). The *Rhizomnium tuomikoskii* (Horik.) T.J Kop. image for Fig. 4C was taken under the same LCSM with fluorescent brightener using a blue laser (λ = 473 nm), and images for Fig. 4A were originally obtained with a polarizing microscope, and then processed in digitized format in Areoana, as explained in Ivanov and Ignatov (2011, 2013).

RESULTS

Observations on early stages of SAP formation in sphagnalean and protosphagnalean moss leaves

Cells in the rapidly dividing cell areas are invariably isodiametric, and usually quadrate. In most cases no differentiation can be observed in such areas (Fig. 3D, I, N), but in *Vorcutannularia* some differentiation is often seen in the areas of smallest cells, where some of them are slightly inflated and paler in colour (Fig. 3F). The area of smallest cells may be totally homogeneous, or sometimes groups of 4 × 4 cells can be discerned within such a mass (Fig. 3N, arrowed).

The apparent cell differentiation usually appears along the distal edge of such areas. Here, cells form conspicuous rows orientated in subtransverse direction to the leaf axis in a fairly

regular manner: one row includes larger cells, usually somewhat inflated and short-rectangular, being usually broader than long. Rows of such cells alternate with rows in which the number of cells is double, and every two cells in this row line up to one larger cell in the neighbouring row (cf. Fig. 3 and scheme in Fig. 2P1). These smaller cells look like they have divided recently, which is suggested by their shapes: the outlines of the cell pairs are rounded and the walls separating cells within each pair are straight. Only very small cells form triads wherein smaller cells line up exactly with the larger one (Fig. 3M). In areas with somewhat larger cells the rows are already offset laterally with respect to the row with the paired smaller cells (cf. Fig. 3O and schemes in Fig. 2P1, P2). In some cases, rows of large cells are longitudinal in orientation instead of transverse (Fig. 3H, L), and in these cases the larger cell rows are displaced towards the leaf apex.

Further cell differentiation beyond these general patterns varies between genera. In protosphagnalean mosses with weakly developed cell dimorphism, e.g. in *Kosjunia* and *Intia*, triads may not differentiate any further in fully developed leaves (Fig. 3H, L, O). Triads are the main unit of the areolation of *Junjagia* (Fig. 3M), whereas in most protosphagnalean mosses the differentiation goes further, up to tetrads (Fig. 3B) or pentads (Fig. 3G), where the SAP cell 1 and SAP cell 2 are darker in colour, forming cell dimorphism similar to that in *Sphagnum* (Fig. 1S3′). At the same time, zones of triads (Fig. 3E, J) are seen between meristematic zones and areas with fully developed cell dimorphism.

Areoana analysis of cell areas and length to width ratio within triads, tetrads and pentads.

The study was conducted on six leaves, four with strongly dimorphic cells and two with laminae composed primarily of hexagonal cells (Fig. 5A–D), but with triads available for SAP cells measurements. Retrospective reconstruction of cell divisions has been done manually by assigning to cell walls the order based on the sequence of cell divisions, in 2194 SAP units with 8976 cells (Table 1). Consistent with expectation, cell size decreases from SAP cell 1 to SAP cell 5, but the last cell is always larger than the one preceding it (Table 1). Another trend is that the more differentiated an SAP unit is, i.e. the farther cell divisions have progressed in that unit, the longer are the cells forming it. For example, in *Protosphagnum* (cf. Fig. 3G) where differentiation often results in pentads, cell length to width ratios are about 3: 1. In species in which SAP development stops at the tetrad stage, cells are shorter, approx. 2: 1, and in leaves where SAP is only locally expressed, while most of the leaf is composed of hexagonal cells (cf. Fig. 6H) and triads are not numerous, the length to width ratios are still shorter, close to 1.5: 1.

Areoana analysis of the SAP expression and distribution in different genera and in different parts of a leaf

The level of SAP expression within a leaf can be evaluated by calculating the number and spatial distribution of substitutions of hexagonal cells by 8 + 5+5 triads and 8 + 7+5 + 4-tetrad
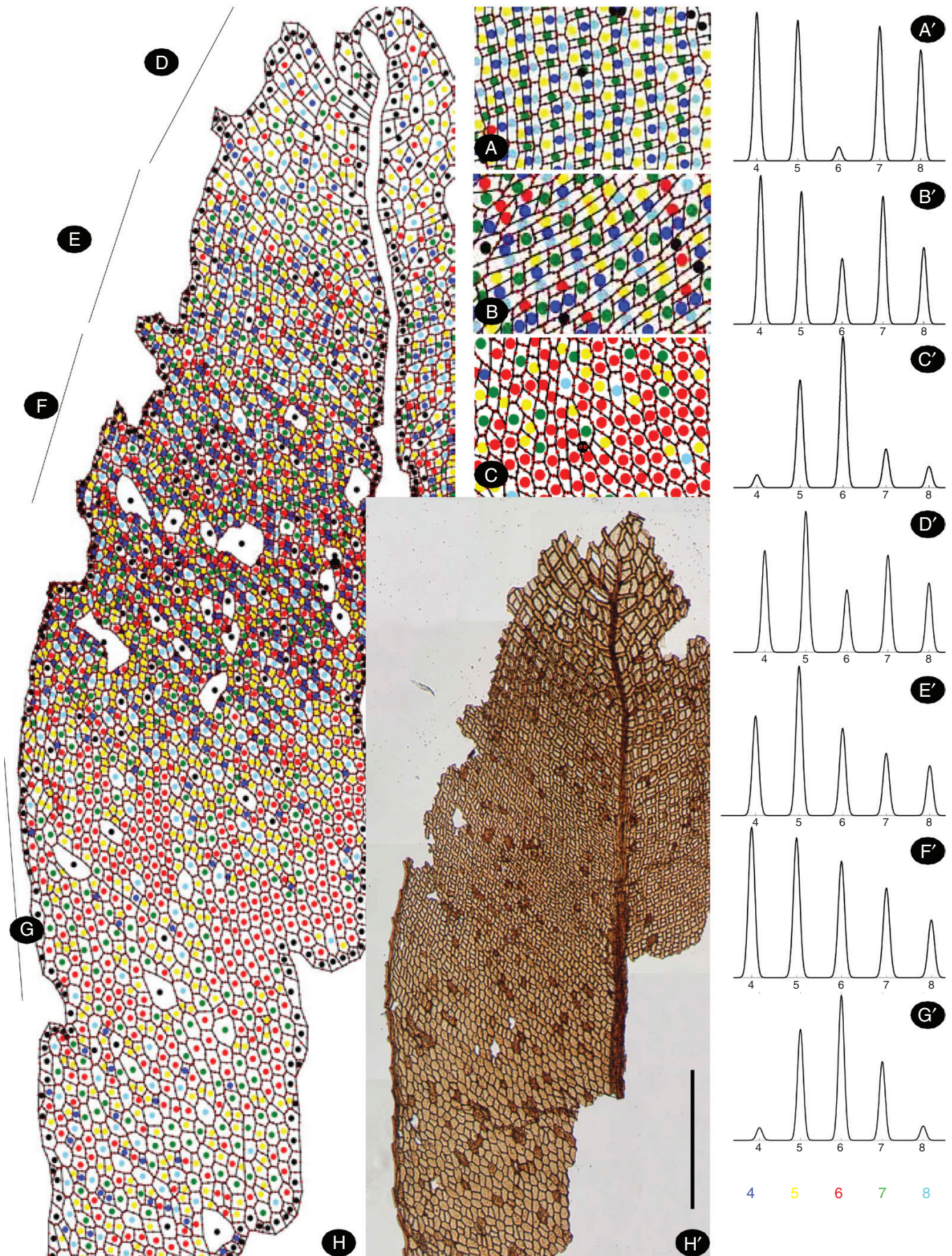
TABLE 1. *Relative areas of the SAP cells (1:2:3:[4]:[5]), as a percentage of the whole SAP-unit, and length to width ratios given as quotients between correspondent cells; numbers of SAP units are given in parentheses; for specimens illustrated in the paper the reference to the figure is given*

Species/specimen/figure	Variable	Pentads	Tetrads	Triads
Protosphagnum 32M_4_6 [not shown]	area	30: 23: 18: 12: 16	32: 25: 18: 24	39: 25: 36
	length: width	3.3: 3.2: 2.9: 3.2: 2.9 ($n = 98$)	3.1: 3.1: 3.2: 2.9 ($n = 247$)	2.8: 3.1: 2.9 ($n = 67$)
Protosphagnum A-41-3_100_1 (part of areolation, Fig. 2G)	area	39: 22: 17: 7: 14	35: 24: 17: 23	37: 26: 36
	length: width	3.0: 2.4: 3.1: 2.7: 3.2 ($n = 9$)	2.8: 3.0: 3.7: 3.1 ($n = 301$)	2.3: 3.6: 3.2 ($n = 270$)
Vorcutannularia 32M_4_35_1 (lower part, Fig. 4F, G)	area	34: 24: 18: 10: 15	37: 25: 16: 22	43: 23: 34
	length: width	2.1: 2.3: 1.7: 2.0: 1.6 ($n = 77$)	1.9: 2.2: 1.7: 1.5 ($n = 267$)	1.8: 1.9: 1.7 ($n = 101$)
Vorcutannularia 32M_4_35_1 (upper part, Fig. 4D, E)	area	31: 21: 18: 12: 18	32: 23: 20: 25	37: 24: 38
	length: width	2.1: 2.3: 1.7: 2.0: 1.7 ($n = 15$)	2.2: 2.3: 1.9: 1.7 ($n = 131$)	2.0: 2.2: 1.7 ($n = 34$)
Vorcutannularia 32M_9_15 (Fig. 3A, B, E, L, M)	area	32: 22: 17: 12: 17	36: 26: 16: 22	43: 24: 34
	length: width	1.9: 2.1: 1.6: 2.1: 1.6 ($n = 6$)	2.0: 2.0: 1.6: 1.5 ($n = 200$)	1.9: 1.9: 1.7 ($n = 120$)
Intia cf. *variabilis* 32M_14_9_5 (Fig. 3H, I)	area	35: 18: 21: 12: 15	–	47: 24: 29
	length: width	1.7: 1.3: 1.5: 2.0: 1.1 ($n = 1$)	–	1.6: 1.5: 1.4 ($n = 73$)
Intia variabilis 32M_2_16_1 (Fig. 3J, K)	area	–	–	50: 22: 28
	length: width	–	–	1.4: 1.6: 1.4 ($n = 110$)

assemblages. The labelling in five colours of cells with 4, 5, 6, 7 and 8 corners (cf. Figs 1 and 2) easily conveys such patterns visually. Modern moss leaves are composed mainly of hexagonal cells (Fig. 4A), and this is also observed in some protosphagnalean mosses, especially in younger leaves (Fig. 5A, B). The protosphagnalean genus *Vorcutannularia* has the smallest number of hexagonal cells, exhibiting maximal cell differentiation, similar in this aspect to *Sphagnum* (Figs 5G, H and 6A). The material studied here provides a series of leaves with increasing SAP expression (Fig. 5). In some *Intia* leaves just one row of triads is developed (Fig. 5C, D), although SAP may be much more developed in specimens representing this genus (cf. Figs 3H and 5E, F). There are scattered areas with SAP in *Intia variablis* (Fig. 5E, F), a species which provides the widest range of variation, all the way to morphotypes with well-developed SAP (Fig. 3H). Finally, large parts of leaves in *Vorcutannularia* are very poor in hexagonal cells (Fig. 5G, H).

The level of SAP expression may vary greatly within a single leaf as seen in Fig. 6, which illustrates one such example and its comparison with 'typical' cell patterns published by Neuburg (1960) (Fig. 6A–C). The distributions of cells with the different number of corners in addition to their labelling in colours (Fig. 6H) facilitates comparison, confirming the similarity of the distal part of this leaf (Fig. 6D) with *Vorcutannularia* and of the basal part (Fig. 6G) with *Intia*. Note that the more homogeneous areolation, with numerous hexagonal cells and the lowest SAP expression, occurs below the zone of smallest and apparently still differentiating cells, whereas the SAP-enriched *Vorcutannularia*-type lamina is seen above that zone. The same distribution of SAP within leaves seems to be the rule, as it is present in several other fragments (not shown). The opposite situation, where SAP is better expressed below the zone of small cells than above it, has not been encountered, although in the *Intia–Protosphagnum* type where the differentiation proceeds from the apical part of leaf (cf. Fig. 3C), the SAP

is usually better expressed in the leaf bases than in the middle portion of leaves.

DISCUSSION

The 'Sphagnoid Areolation Pattern', or SAP, is identical in its principal structure in modern *Sphagnum* and Palaeozoic protosphagnalean mosses, despite having developed considerably differently in these two groups. In *Sphagnum*, SAP is formed as a result of unequal oblique cell divisions (Fig. 1), as has been shown in detail by Schnepf (1973). In contrast, in protosphagnaleans cell divisions appear to have been equal and the SAP formed as a result of subsequent cell growth (Fig. 2P1–P3, PD). It is not possible to study ontogenesis of fossil material in as much detail as in living plants, so our reconstructions are necessarily based on comparisons of stages that are assumed to be sequential because of relative position, starting from the leaf base and progressing towards the middle part of the leaf (cf. one leaf in Fig. 3A, D, I and another in Fig. 3B, E, F, J). In modern mosses, these developmental stages can also be traced within an individual leaf, from its base to apex (Pottier, 1925).

Formation of triads seems to be a crucial stage, because the SAP pattern is formed as early as this stage in both protosphagnalean mosses (Fig. 2P1, P2) and *Sphagnum* (Fig. 1S2). Triads are usually absent in the zone of active cell division in the basal part of the leaf (Fig. 3D, N), although sometimes cells are arranged in triads in this region, such as in the *Vorcutannularia* leaf base (Fig. 3J). Our material includes more than ten specimens in which the basal leaf portion has an extensive area of conspicuously equal cells 4–5 µm in size, i.e. as small as the smallest cells we have seen in the corresponding meristematic zones in leaves of modern mosses. Based on this, we can infer that at least at this stage, cell divisions in protophagnalean moss leaves are equal. The distal edges of these small-celled areas

FIG. 6. Areolation patterns and graphs showing relative distributions of cells with four to eight corners in digitized images from (A) *Vorcutannulatia pliacta* (Neuburg, 1960, fig. 70–1), (B) *Protosphagnum nervatum* (Neuburg, 1960, fig. 75–1), (C) *Intia variabilis* (Neuburg, 1960, fig. 7–2)), (D–G) different parts of the leaf of *Vorcutannularia* sp., specimen 32M_4_35_1; the whole leaf is shown both as digitized areolation (H) and micrograph (H′). Each graph in the column on the right (A′–G′) shows the relative distribution of cells with four, five, six, seven and eight corners (*y*-axis is in arbitrary units, showing distribution smoothed by a Gaussian function). Scale bar for H′ = 0.5 mm. For colour legend see Fig. 1.

display cell differentiation towards triad formation even before cell size has reached 10 µm (Fig. 3N, inset).

The youngest triads along the distal edge of meristematic zones were observed always associated in rows of somewhat inflated, transversely rectangular cells (Fig. 3M, arrows). These rows alternate with rows containing double the number of cells, in which the cells are about half the size and every other one lines up with a transversely rectangular cell of the next row. At least in some cases this formation of a T-pattern (Figs 2P1 and 3M) takes place without any displacement, although such cases are rare, and usually the transversely rectangular cell is offset with the formation of octagonal cells, i.e. an already developing SAP (Figs 2P2 and 3O).

This pattern is very regular and transverse rows of transversely rectangular cells often cross the whole distance from costa to leaf margin (Fig. 3I, K, L, O). Considering that this pattern may sporadically appear in leaves that otherwise lack SAP almost completely (Fig. 5C), we suggest that cells in these rows are highly conductive. Importantly, these rows unite and comprise highly homogeneous cells with putatively different ontogenetic histories, i.e. formed in different cells packets. During development, all moss leaves go through stages when the cell divisions result in cell packets of 4 × 4, 8 × 8 and up to 64 × 64 cells (Frey, 1970; Bopp, 1984; Donskov, 2015), in which the orientation of cell divisions alternates regularly between transverse and longitudinal. Such cell packets are easy to observe in juvenile leaves (Fig. 4C), but also appear contrasted in mature leaves, for example in Areoana as areas rich in hexagonal cells (Fig. 4A). In protosphagnalean mosses, cell packets are only moderately apparent, but the 4 × 4 cell packets can be recognized at places in basal meristematic zones (Fig. 3N, arrows). In modern mosses, cell packets are at least slightly offset (Fig. 4A, C) and, as a result, straight rows of regularly shaped cells as in protosphagnalean mosses are never observed. The conspicuous alignment of cells in rows clearly formed by cells from different 4 × 4 cell packets, as explained above, is noteworthy and suggests the presence of a morphogenetic factor (phytohormone?) facilitating row formation, although there are no ways to test this hypothesis at present.

An intriguing question is whether the T-pattern position is somehow predefined or if any two neighbouring cells, under certain circumstances, may combine to form it. The latter scenario is different from that present in *Sphagnum*, where the T-pattern develops in a cell which has already undergone uneven oblique division (Fig. 1S1, S2). However, there are at least two reasons to infer that T-pattern formation in protosphagnalean mosses was more opportunistic, and might involve cells independently of sequences of cell division.

Firstly, the direction of the rows changes within the leaf, being transverse in some areas and longitudinally orientated in others (Figs 3A, H, O, 4D, 5G, H and 6H). Furthermore, it is not rare to find asymmetry in leaves wherein longitudinally orientated rows predominate on one side of the costa, and transverse rows on the other (Figs 3A and 5G, H). However, changes in the direction of the rows often occur in smaller areas following no apparent rule (Figs 3H and 4D).

Secondly, in places T-patterned triads are formed singly among hexagonal cells or form very short rows of two T-patterned triads (Fig. 5A–F). The broad spectrum of observed variants of the T-pattern distribution across the leaf lamina

seems to indicate that the conjoining of cells into a triad is determined very late.

The ability to direct development toward SAP or otherwise to hexagonal areolation is especially conspicuous in some leaves of *Vorcutannularia* sp. (Fig. 6H). We observed several more or less complete leaf fragments that include such putatively meristematic small-celled zones in the mid-leaf region and all have the same type of differentiation. Cells above such zones are developing SAP, whereas cells below these zones are largely hexagonal. This results in a high diversity of morphotypes, only incompletely illustrated here in Figures 3–6. Note also that morphotypes lacking SAP are not discussed or illustrated and only one example is shown here (Fig. 5A, B).

Interestingly, a very different ontogenetic pathway in sphagnalean and protosphagnalean mosses results in a largely identical pattern of cell arrangement, the SAP, which seems to be the only alternative to the hexagonal cell areolation.

Both pathways are similar in that the smallest cell within the SAP unit is the one produced by the last division of the developmental sequence, regardless of whether these are triads, tetrads or pentads (Table 1). This is consistent with the conclusion that the idioblast cells originate from the last division in the cell-packets, and is a common feature of both flowering plants (e.g. *Monstera*) and filaments of the cyanobacterium *Anabaena* (Barlow and Lück, 2008). In this case, the SAP similarity in sphagnalean and protosphagnalean mosses may be caused by this more general rule.

CONCLUSIONS

Sphagnalean and protosphagnalean mosses are both characterized by a specific lamina cell patterning, the SAP, which is not known in any other bryophytes. In sphagnaleans, the SAP forms as a result of two unequal and oblique cell divisions in early stages of development. In contrast, in protosphagnaleans cell divisions appear to have been equal and the SAP formed as a result of subsequent cell growth. Moreover in protosphagnalean mosses the SAP started with T-pattern formation from two cells by division of one of them, and subsequent lateral displacements of the undivided cell against a pair of smaller cells with which it lines up. This pathway of SAP formation allows for the beginning of its differentiation at later stages in leaf development, thus providing the ability to switch ontogenetic pathways from SAP to the variant common to all other mosses, i.e. hexagonal cell formation. Thus, SAP may be expressed in the whole leaf or only in parts of it, or it can be totally absent, with various combinations in different genera. This new understanding of the ontogeny allows us to re-evaluate the systematic significance of such diagnostic characters in Palaeozoic protosphagnalean mosses, showing that their anatomical similarity to *Sphagnum* is less meaningful in terms of evolutionary relationships than previously thought.

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