

RESEARCH IN CONTEXT

## Hydathodes in ferns: their phylogenetic distribution, structure and function

Klaus Mehltreter<sup>1,2,\*</sup>, Hanna Wachter<sup>2</sup>, Christophe Trabi<sup>2</sup>, Weston Testo<sup>3,4</sup>, Michael Sundue<sup>5</sup> and Steven Jansen<sup>2</sup>

<sup>1</sup>Red de Ecología Funcional, Instituto de Ecología, A.C., Xalapa 91073, Veracruz, México, <sup>2</sup>Institute of Systematic Botany and Ecology, Ulm University, D-89081 Ulm, Germany, <sup>3</sup>Department of Biological and Environmental Sciences, University of Gothenburg, 40530 Goteborg, Sweden, <sup>4</sup>Botanical Research Institute of Texas, Fort Worth, TX 76107, USA and <sup>5</sup>The Pringle Herbarium, Department of Plant Biology, University of Vermont, Burlington, VT 05405, USA

\*For correspondence. E-mail [kmehltreter@gmail.com](mailto:kmehltreter@gmail.com)

Received: 27 March 2022 Returned for revision: 24 May 2022 Editorial decision: 8 June 2022 Accepted: 11 June 2022  
Electronically published: 13 June 2022

- **Background and Aims** Ferns are the second largest group of vascular plants and are distributed nearly worldwide. Although ferns have been integrated into some comparative ecological studies focusing on hydathodes, there is a considerable gap in our understanding of the functional anatomy of these secretory tissues that are found on the vein endings of many fern leaves. In this study, we aimed to investigate the phylogenetic distribution, structure and function of fern hydathodes.
- **Methods** We performed a global review on fern hydathodes and their phylogenetic distribution, carried out an ancestral character state reconstruction, and studied the structure, guttation and elemental composition of salt residues of eight species, and the diurnal patterns of xylem pressure of two species.
- **Key Results** Hydathodes are known from 1189 fern species, 92 genera and 19 families of 2 orders, Equisetales and Polypodiales. Stochastic character mapping indicated multiple gains and losses of hydathodes at the genus level, occurring especially during the last 50 million years of fern evolution. Hydathodes were located on the adaxial leaf surface and characterized by a cytoplasm-rich, pore-free epidermis, and became functional for several weeks after nearly complete leaf expansion. In two species, positive xylem pressure built up at night, potentially facilitating guttation. Guttation fluid was rich in Ca and often Si, but also contained P, Mg, Na and Al.
- **Conclusions** Stochastic character mapping and the structural and functional diversity of hydathodes indicate multiple origins, and their presence/absence in closely related taxa implies secondary losses during fern evolution. Positive xylem pressure and high air humidity play an important role as drivers of guttation. Hydathodes may contribute to the regulation of leaf nutrient stoichiometry by the release of excessive compounds and minerals other than waste products, but the presence of essential chemical elements in salt residues also indicates possible leakage.

**Key words:** *Aglaomorpha*, *Campyloneurum*, ferns, guttation, hydathodes, leaf venation, *Nephrolepis*, *Phlebodium*, plant nutrients, positive xylem pressure, *Pyrrhosia*, salt glands.

### INTRODUCTION

Vascular plants absorb water and dissolved minerals through their root hairs and distribute them throughout their body with the help of their vascular tissues. In the leaves, vascular tissues are often visible as numerous parallel, branched or netted veins that end at the leaf margin or run into a marginal vein. However, some plant species have enlarged vein endings reaching an epithem, a subepidermal specialized tissue, connected to the leaf surface or the leaf margin, which can secrete liquids through the epidermis or stomata-like pores. Because of the composition of the released liquids and the anatomy of the secretory tissues, these were named by Fahn (1979, 1987, 1988) nectaries, salt glands or hydathodes.

The term ‘hydathode’ has been used in the past to refer to the following structures: (1) water pores or water glands, depending on the presence or absence of pores (German: Wasserspalten,

Wassergübchen, Wasserdrüsen; Haberlandt, 1894); (2) lime dots when visible salt residues remain after the evaporation of the water (e.g. Holttum, 1954); or (3) conspicuously thickened vein endings. In our review, we have included all these definitions, but our experiments were focused specifically on lime dots. In angiosperms, the derivation of pore-bearing hydathodes from stomata has been suggested (Evert, 2006). However, pore-free hydathodes, as they have been reported for ferns, might have had different developmental origins because of their distinct location on the leaf surface or leaf margin and differences in the histological structure of the underlying epithemic tissue (Haberlandt, 1894). Hydathodes are often differentiated from the surrounding tissues by their compact, small-sized, plasma-rich subepidermal epithemic and epidermal cells, and are often connected with short, widened tracheids to the vascular system (Smith, 1995; de la Sota *et al.*, 2000). Hydathodes trigger the connection to the minor veins during leaf expansion because

they release auxin, which induces the basipetal formation of tracheary elements and their connection to the developing vascular system (Davies, 2010).

The main function attributed to hydathodes is guttation, the release of xylem sap containing dissolved substances such as salts, sugars and amino acids (Goatley and Lewis, 1966; Singh, 2016). Several authors distinguished passive and active hydathodes depending on their passive role as mere pores or filters through which water is forced by positive xylem pressure (Dieffenbach et al., 1980) or their active role as water- and salt-releasing glands by a still unknown energy-consuming process in the hydathode cells (Wild and Schmitt, 2012). In both cases, guttation supposedly maintains a minimum xylem flow to allow a continuous supply of mineral nutrients at night when photosynthesis is suspended and transpirational pull is low or absent. Because hydathodes can also occur in some aquatic plants (Ingrouille and Eddie, 2006) and some studies detected toxic elements such as arsenic in the salt residues (Cantamessa et al., 2016), an alternative function has been proposed, which suggests that hydathodes allow the exudation of an excess of secondary metabolic waste products. In drier, terrestrial and epiphytic habitats with nocturnal fogs, the function of hydathodes might even have become inverted and used for foliar water uptake (Haberlandt, 1894; de la Sota, 1966; Limm et al., 2009; Martin and van Willert, 2000), comparable to the function of foliar scales in the resurrection fern, *Pleopeltis polypodioides* (John and Hasenstein, 2017). The phenotypic expression of hydathodes seems to be transient in some closely related fern clades, developing and disappearing quickly during their evolutionary history, as has been shown specifically for grammitidoids (Ranker et al., 2004; Sundue et al., 2010a; Kessler et al., 2011; Sundue et al., 2012). In hybrids, hydathodes can take on intermediate morphologies. For instance, the hybrid between the hydathode-bearing *Aglaomorpha coronans* and hydathode-less *Aglaomorpha rigidula* does not possess hydathodes, but instead exhibits enlarged vein tips (Hoshizaki, 1991).

Ferns originated as early as the late Silurian (Testo and Sundue, 2016), radiated and adapted to many habitats in angiosperm-dominated forests during the Cenozoic (Schneider et al., 2004; Mehlreter et al., 2010), and are the second largest extant group of vascular plants on Earth, comprising ~10 578 species (PPG I, 2016). They play an important role in many tropical ecosystems, but ecological research such

as the investigation of their water and mineral balance has been widely neglected when compared with seed plants. In ferns, ecophysiological research in the last two decades has focused on their vascular anatomy (Hernández-Hernández et al., 2012), biomechanics (Mahley et al., 2018), functional constraints (Pittermann et al., 2011) and hydraulics (Watkins et al., 2010; Brodersen et al., 2014; Brodribb et al., 2016; Klepsch et al., 2016), mainly related to stomatal regulation, desiccation tolerance, and drought avoidance (Campany et al., 2021), but very little attention has been paid to the phylogenetic distribution, morphology and function of specific secretory tissues such as nectaries (Mehlreter et al., 2022), salt glands and hydathodes (Fahn, 1987, 1988).

In ferns, hydathodes have been reported for Blechnaceae, Cyatheaceae, Dryopteridaceae (genera *Elaphoglossum*, *Megalastrum*, *Stigmatopteris*; Sundue et al., 2010b; Moran and Labiak, 2016), Nephrolepidaceae (Smith and Kessler, 2018), Polypodiaceae (especially grammitidoids; Sundue, 2010a, 2010b, 2013; Sundue et al., 2010a, b; Lagoria et al., 2018) and Woodsiaceae. Guttation in ferns was suggested to be a passive mechanism driven by positive xylem pressure; the latter has been confirmed for few fern species (Sperry, 1983; Ewers et al., 1997; Fisher et al., 1997; Holmlund et al., 2020). Although positive xylem pressure is typically described in the literature as ‘root pressure’, the source of the positive pressure is typically not known and may not be limited to roots (Schenk et al., 2020). Therefore, we use the more general term ‘positive xylem pressure’ in this manuscript. The solutes of the hydathode fluid can precipitate and form conspicuous white spots on the dry leaf surface (Figs 1 and 2). These salt residues were supposed to consist of calcium carbonate (Holttum, 1954) but their composition has never been analysed in detail. Because hydathodes of many ferns do not produce lime dots (i.e. are non-calcareous), they may only operate under specific environmental conditions, such as high air humidity or high soil cation content. Although hydathodes have attracted the attention of several researchers since the late 19th century (Gardiner, 1883; Haberlandt, 1894), little progress has been made concerning their phylogenetic distribution, anatomical structure and function in ferns. Consequently, the aim of our study was to investigate (1) the phylogenetic distribution and ancestral character state of hydathodes in ferns, (2) their arrangement in correlation with vein patterns and structure, (3) the positive xylem pressure and diurnal patterns of guttation, (4) the chemical composition



FIG. 1. Fern hydathodes with guttation droplets on upper leaf surfaces. (A) *Nephrolepis cordifolia*; (B) *Campyloneurum brevifolium*; (C) *Phlebodium aureum*. Scale bars = 5 mm.

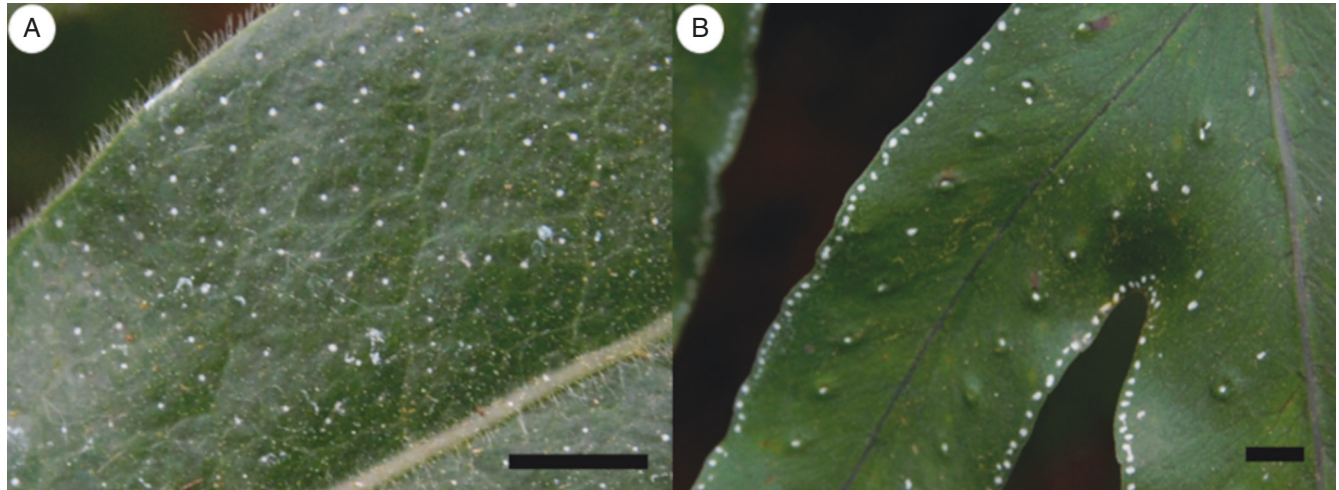


FIG. 2. Hydathodes with salt residues (=lime dots). (A) *Aglaomorpha pilosa*; (B) *Phlebodium aureum*. Scale bars = 5 mm.

of salt residues, and (5) the variation of all studied variables across different fern species.

## MATERIALS AND METHODS

### *Phylogenetic distribution of hydathodes in ferns*

To understand the occurrence of hydathodes in an evolutionary context, we mapped the distribution of hydathodes and estimated ancestral character states on a genus-level fern phylogeny using R version 3.6.3 (R Core Team, 2020). This phylogeny was modified from the time-calibrated phylogeny of Testo and Sundue (2016) and was generated by using the `drop.tip` function in the R package `ape` version 5.4-1 (Paradis *et al.*, 2004; Paradis and Schliep, 2019) to retain a single representative taxon from a total of 273 fern genera. We then scored the occurrence of hydathodes in these 273 genera based on a review of online databases, literature, and observations in the living fern collections at the Botanical Gardens of Berlin, Bochum, Munich, Tübingen, Ulm (all Germany) and Kew (London, UK). A literature search in online databases (Web of Science) was performed with the combined keywords ‘hydathode’ AND ‘fern’. In printed floras and monographs at the Natural History Museum, London, the book index and the verbal descriptions of genera and species were searched for the term hydathode but also under the terms of enlarged or widened or clavate vein endings, lime dots, lime scales, calcareous or cretaceous scales. Floras included Holttum (1954), Morin (1993), Davidse *et al.* (1995), McCarthy (1998), Mickel and Smith (2004) and Zhengyi and Raven (2013) and monographs such as Kramer and Green (1990). Any evidence for hydathodes within a fern genus was further investigated and confirmed targeting specific monographs of the corresponding genus (e.g. León, 1993; Hovenkamp, 1998; Gasper *et al.*, 2016) and images in online herbarium databases (e.g. TROPICOS). Because many monographs and floras do not provide information on hydathodes for each reported species, we scored and analysed hydathodes at genus level (but see Supplementary Data File S1 for a working list of hydathode-bearing species). Two character states were scored: (1) hydathodes absent in all

species; and (2) hydathodes (or enlarged vein endings) present in at least one species. Seventy-four genera for which hydathode presence/absence is unknown were excluded from further analyses. The detailed data set that distinguishes between hydathodes and enlarged vein endings, and between genera with hydathodes in at least one species or at least one species, is provided and mapped (Supplementary Data File S2). For further analyses, however, we chose the presence/absence data set as it appropriately captures most known variation in hydathode appearance in ferns and it could be scored confidently with the available data. The distribution of these character states on the phylogeny was visualized using the `plotTree` and `add.simmap.legend` functions in the R package `phytools` version 0.7.70 (Revell, 2012) and the `tiplabels` function from `ape`.

To infer ancestral character states, we used the 199-tip phylogeny and corresponding character state information applying the `ace` function in `ape` using the maximum likelihood (ML) method. The reconstruction was visualized using the `phytools` functions `plotTree` and `add.simmap.legend` and the `ape` functions `nodelabels` and `tiplabels`. We conducted stochastic character mapping on the 199-tip phylogeny using the `make.simmap` function in `phytools`. The aim of both ancestral character state reconstruction analyses was to obtain estimates for the minimum number of gains/losses of hydathodes in ferns at genus level. An analysis at species level was not feasible because of too many missing data, and the lack of molecular data for ~60 % of fern species (Testo and Sundue, 2016). We ran the analysis with 500 iterations and summarized the output with the `countSimmap` function in `phytools`. All tree files, character matrices and R code are available at <https://github.com/wtesto/FernHydathodes/>.

### *Study species and cultivation*

The anatomy and function of hydathodes were studied in eight species of two families: Nephrolepidaceae (*Nephrolepis*) and Polypodiaceae (*Aglaomorpha*, *Campyloneurum*, *Niphidium*, *Phlebodium*, *Pyrrosia*) to cover a variety of ferns within the order Polypodiales from the Neotropics as well

as from the Palaeotropics. All selected species were producing salt residues on their simple to pinnate leaves of variable size (Table 1). Living material was generously made available from the Botanical Gardens of Berlin, Bochum, Munich, Tübingen and Ulm, and cultivated in the tropical greenhouses of the Botanical Gardens at Ulm University in a mixture of peat-growing medium (TKS2) with 5 % ground pumice. To stimulate guttation, plants were well-watered and kept in humid chambers (>90 % relative humidity) within the greenhouses under natural light conditions and at a mean temperature of 24 °C.

#### Leaf venation and anatomical structure of hydathodes

To study the location of hydathodes in relation to leaf venation, fresh leaves were fixed in 70 % ethanol, bleached first with 5 % NaOH for 1–7 d, then with 4.5–5.5 % NaClO for 20 s or up to 10 min until they became transparent (depending on the species), and finally stained with safranin (Vasco *et al.*, 2014).

For light microscopy, pieces of 1 × 2 mm size with a hydathode in the centre were cut out of a healthy fern leaf and first fixed in glutaraldehyde for 12–24 h, then rinsed and washed with buffer, followed by post-fixation in osmium tetroxide for 2 h at room temperature, rinsing in buffer, washing with water, dehydration in an acetone series (three times), infiltration with Epon–acetone, immersion in fresh 100 % epon (twice) and embedding in Epon (Tizro *et al.*, 2019). For additional observations and pictures, dry leaf pieces were sputter-coated with gold (2 min) and then observed under a Phenom XL (Nano Science Instruments, AZ, USA) desktop scanning electron microscope (SEM).

#### Xylem pressure and guttation

We measured the xylem pressure in two of the eight fern species (*Nephrolepis cordifolia* and *Phlebodium aureum*). The round stipules of these species had a length and diameter that was sufficient to connect them to silicon tubes without damaging

the base of the lamina and causing leakage. For each species, we measured three plants for at least 48 h and averaged the maximum and minimum values per individual and species. To determine the xylem pressure of these ferns with actively guttating hydathodes, we mounted a pressure sensor with a silicon tube to a recently decapitated leaf petiole base. A pressure sensor (Omega 26 PCDF6D) was connected via an analogue–digital converter (ADS1115, 16 bits, I2C) on an Arduino Uno microcontroller powered by a 12-V battery connected via a 10-V voltage regulator. Environmental data (temperature, relative humidity and atmospheric pressure) were monitored in parallel with a triple-sensor BME-280. Measurements were taken every 5 min for at least 48 h. Data were collected on an SD card datalogger with an RTC (5 V) and at the end of the measurements were transferred as a .txt file from the MicroSD card to a tabulating datasheet. Mean values of maximum and minimum xylem pressure were compared between the two species by means of a *t*-test in SigmaStat 3.5 (SysStat Software).

To determine possible day–night cycles of hydathode activity (= release of guttation fluid), we took time-lapse pictures with a digital camera (Nikon Coolpix 8400) in a macro mode every 10 min for at least 24 h, converted these into movies at 30 frames per second, and edited the best with Camtasia Studio 8 (TechSmith®), inserting explanatory subtitles. For time-lapse pictures, plants were well hydrated and kept in the greenhouse at temperatures between 20 and 25 °C under high air humidity (>90 %).

#### Elemental analysis of salt residues

To analyse the composition of salt residues of active hydathodes, small hydathode-bearing pieces of three leaves (<5 × 8 mm) of each species were air-dried (the application of any solutions might have modified the salt compositions), mounted on a grid and coated with a 3-µm-thin C-layer (graphite) using a MED010 apparatus (Balzers Union, Balzers, Fürstentum Liechtenstein). The samples were observed under a Hitachi S-5200 FE-SEM at 20 kV, 10 µA, which was connected to an EDX unit (HIT S-65 136-5 Detecting Unit, Edax Business

TABLE 1. *Characteristics and distribution of the eight fern species.*

Family/species	Life form	Leaves*	Natural distribution
<b>Nephrolepidaceae</b>			
<i>Nephrolepis brownii</i> <sup>2</sup>	(ep), t	Pinnate; 70–130 cm	Palaeotropics
<i>Nephrolepis cordifolia</i> <sup>1,2,4</sup>	(ep), t	Pinnate; 40–120 cm	Palaeotropics
<b>Polypodiaceae</b>	ep	Pinnatifid; 50–80 cm	Moluccas, Philippines, Sulawesi
<i>Aglaomorpha pilosa</i> <sup>3</sup>			
<i>Campyloneurum brevifolium</i> <sup>4</sup>	ep, t	Simple; 40–120 cm	Neotropics
<i>Campyloneurum xalapense</i> <sup>1,4</sup>	ep	Simple; 25–90 cm	Neotropics
<i>Niphidium crassifolium</i> <sup>1,4</sup>	ep, li, t	Simple; 5–40 cm	Neotropics
<i>Phlebodium aureum</i> <sup>5</sup>	ep, li	Pinnatifid; 30–100 cm	Neotropics
<i>Pyrrosia polydactyla</i> <sup>6</sup>	ep, li, t	Pedate; 15–40 cm	Taiwan, Korea

Data source: \*own observations; <sup>1</sup>Davidse *et al.* (1995); <sup>2</sup>Hovenkamp and Miyamoto (2005); <sup>3</sup>Janssen and Schneider (2005); <sup>4</sup>Mickel and Smith (2004); <sup>5</sup>Morin (1993); <sup>6</sup>Zhengyi and Raven (2013).

Life form: ep, epiphytic; li, lithophytic; t, terrestrial.

Unit Ametek GmbH, Wiesbaden, Germany) using the software Genesis Spectrum (Version 5.211, Edax Inc., Mahwah, USA). Elementary detection was performed at a magnification of  $\times 2000$  with an elemental analyser (Edax) for 200 s. Identified elements included Na, Mg, Al, Si, P, S, Cl, K, Ca, Mn, Fe and Zn. Measurements were performed by entirely focusing on salt residues, while clean, undamaged epidermal surfaces served as control. For each species, we measured three samples of different plants. Salt residues were also tested with 0.1 N HCl, which reacts with and dissolves calcium carbonate and calcium oxalate, and 5 %  $\text{CH}_3\text{COOH}$  (acetic acid), which dissolves calcium carbonate but not calcium oxalate.

## RESULTS

### *Phylogenetic distribution and evolution of hydathodes in ferns*

As a result of the literature review, we found evidence for hydathodes in at least 1189 species from 92 genera and 19 of 48 fern families (Supplementary Data File S1). Except for Equisetaceae, all families with hydathode-bearing species belonged to the derived order Polypodiales. Although hydathodes are a fixed trait of 42 genera (e.g. *Campyloneurum*, *Equisetum*, *Melpomene*, *Nephrolepis*), their presence may vary in some genera (e.g. *Alansmia*, *Pyrrosia*) and can occur with conspicuous lime dots (= cretaceous, calcareous) or be inconspicuous without lime dots. In 35 genera (e.g. *Asplenium*, *Blechnum*, *Elaphoglossum*) hydathodes were only present in some species, and in 15 genera (e.g. *Dryopteris*, *Microlepia*, *Pellaea*) there were reports of enlarged vein endings without any signs of salt residues.

An ancestral character state reconstruction showed the independent gain of hydathodes in several fern lineages such as Pteridaceae and Blechnaceae, especially during the last 50 million years, and many secondary losses of hydathodes in Polypodiaceae (Fig. 3). A stochastic mapping analysis on presence/absence data (Supplementary Data File S3) supports a minimum number of character transitions of  $39 \pm 5.88$  (mean  $\pm$  s.d.) gains and  $47 \pm 7.72$  losses of hydathodes at genus level.

### *Anatomical structure of hydathodes*

Leaves of the eight species studied had a free (e.g. Nephrolepidaceae) or netted (e.g. Polypodiaceae) leaf venation, the latter frequently with free vein endings in each areole (an area enclosed by netted veins; Fig. 4A–C). The position of hydathodes depended directly on the location of free vein endings on the adaxial leaf surface (Fig. 4), often situated directly opposite the placenta, with the sorus-forming tissue on the lower leaf surface (Fig. 4B). Consequently, hydathodes could appear in one (Fig. 4D) to several rows (Figs. 4A–C) between midvein and leaf margin. Hydathodes accumulated salt residues in at least some developmental stages in both sterile and fertile leaves. Hydathodes were characterized by compact, cytoplasm-rich epidermal cells and the lack of epidermal pores or stomata. The secretory tissue (epithem) contained vascular bundles with xylem and phloem tissue, had no intercellular spaces, and was surrounded by an endodermis-like tissue

(Fig. 5). Epidermal cells of the hydathodes were smaller, had straight cell walls and often a thinner or no cuticle when compared with the considerably larger surrounding epidermal cells with undulating cell walls and thick cuticle (Figs 5 and 6). In most species hydathodes were flat on the leaf surface, but in *Pyrrosia polydactyla* hydathodes were deeply sunk into the epidermis (Fig. 6H), whereas in *Phlebodium aureum* (Figs 2B and 5A) the hydathode-surrounding tissues were raised and the hydathode itself was impressed in the centre.

### *Ontogeny, positive xylem pressure and guttation*

In all eight fern species, hydathodes became functional and exuded liquids when young leaves expanded to at least 80–90 % of their final size (Fig. 1). Their active phase lasted for 1–3 months until guttation ceased. In *Aglaomorpha pilosa* and *P. aureum*, even older leaves may still guttate, as can be inferred from the increasing diameter of their lime dots in older leaves (Fig. 2).

The two measured fern species, *P. aureum* (Fig. 1C) and *N. cordifolia* (Fig. 1A), both had a maximum positive xylem pressure, of  $33.4 \pm 1.15$  and  $53.5 \pm 9.77$  kPa, respectively, at night, which differed significantly between the two species ( $t = -3.538$ , d.f. = 4,  $P = 0.024$ ). During the day, the average minimum xylem pressure decreased to similar values of  $-11.9 \pm 7.83$  kPa (*P. aureum*) and  $-13.5 \pm 10.21$  kPa (*N. cordifolia*;  $t = 0.209$ , d.f. = 4,  $P = 0.845$ ). The positive xylem pressure built up in the afternoon and during the night when temperatures dropped, and air humidity levels rose above 94 %. In the morning xylem pressure fell to negative values between  $-2$  and  $-20$  kPa together with the decline in air humidity (Fig. 7).

Time-lapse photography revealed that both species released hydathode fluids between midnight and 0600 h, when air humidity was  $>94$  % (Figs 1 and 2, Supplementary Data File S4). However, hydathode fluid evaporated during the day because of the increasing temperature, declining air humidity and negative xylem water potentials associated with photosynthetic activity (Fig. 4C–D).

### *Elemental analysis of salt residues and hydathode fluid*

The species analysed had hydathodes with salt residues that were predominantly made up of Ca (47–62 %) and Si (5–37 %), but also P (0.8–14.6 %), Mg (4.5–14.6 %), Na (0.1–5.4 %) and Al (0–3.8 %) (Fig. 8). Minor elements ( $<4$  %) were S, K, Zn, Mn, Cl and Cu. On the other hand, control measurements of the epidermis without any salt residues detected mainly Ca ( $30.6 \pm 12.3$  %, mean  $\pm$  s.d.), K ( $25.2 \pm 15.4$  %), Cl ( $11.0 \pm 8.0$  %), Mg ( $9.8 \pm 6.5$  %) and P ( $8.0 \pm 4.9$  %), but also contained Si ( $6.4 \pm 7.1$  %), S ( $4.9 \pm 3.6$  %), Na ( $2.8 \pm 3.8$  %), Zn ( $0.2 \pm 0.3$  %), Mn ( $0.2 \pm 0.3$  %) and Cu ( $0 \pm 0.1$  %). However, differences among species were notable, dividing them into at least two distinct functional groups, one with low Si concentrations ( $<10$  %; *Campyloneurum brevifolium*, *Campyloneurum xalapense*, *P. aureum*) and another group of the five remaining species with elevated Si concentrations (17–37 %). Salt residues

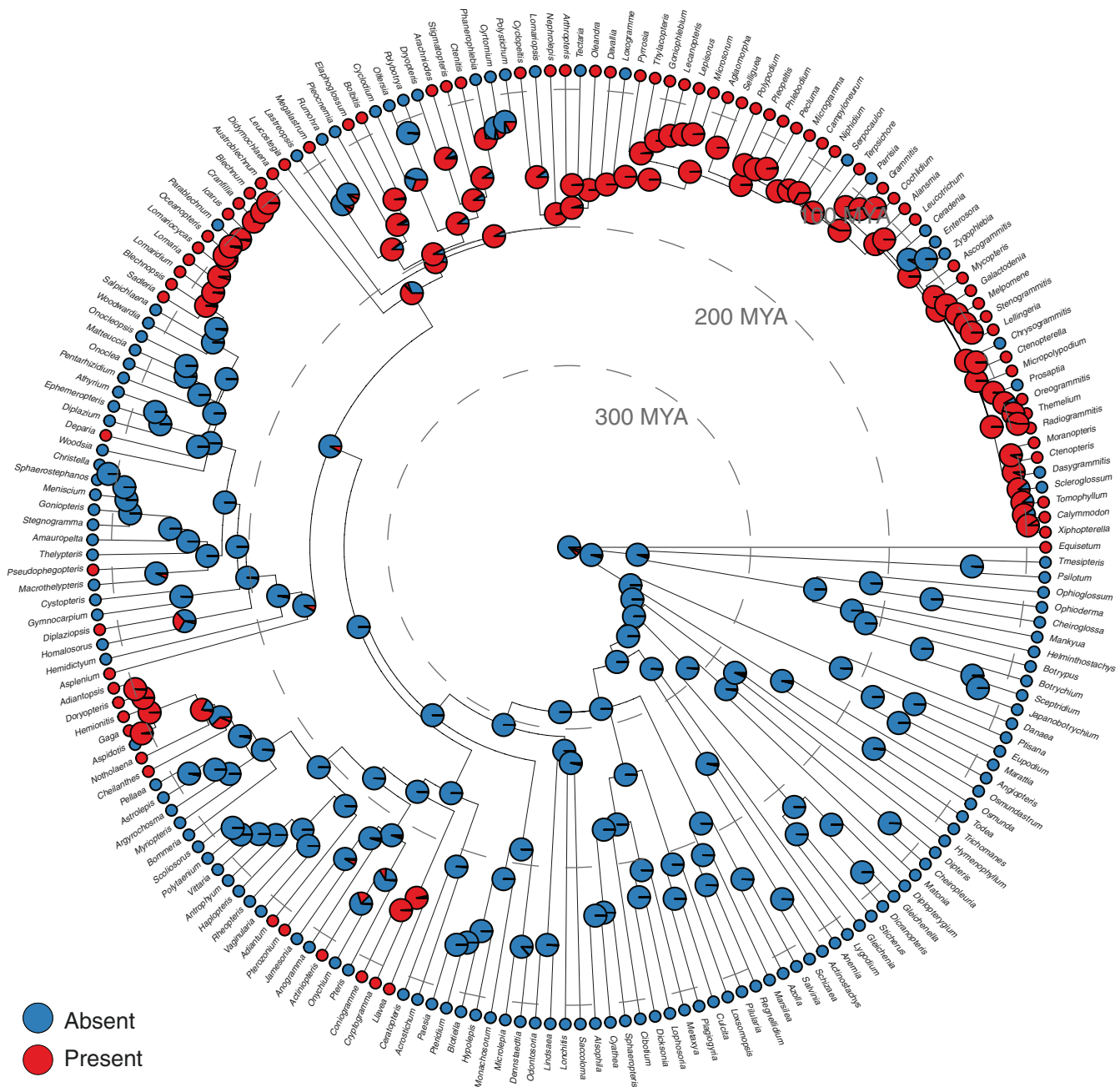


FIG. 3. Fern phylogeny of 199 genera showing results of ML ancestral character state reconstruction of hydathode occurrence.

of some species contained considerable amounts of the valuable essential elements, such as P and Mg, whereas *A. pilosa*, *Nephrolepis brownii* and *Niphidium crassifolium* also contained 2.1, 3.8 and 2.0 % of Al, respectively, indicating some possible release of excess toxic minerals. Differences among species within the same genus (e.g. *Campyloneurum* and *Nephrolepis*) were minor (Fig. 8). Reactions of salt residues with 0.1 N HCl and 5 % CH<sub>3</sub>COOH of all species were more intense (more CO<sub>2</sub> gas bubble formation) with hydrochloric acid than with acetic acid, indicating the presence of calcium carbonate, but also the possible presence of calcium oxalate, which only reacts with hydrochloric acid.

## DISCUSSION

### Phylogenetic distribution and evolution of hydathodes in ferns

Hydathodes were present in 92 fern genera and >10 % of all 10 578 known fern species (PPG I, 2016) when defining hydathodes in a broad sense. Their character state reconstruction, phylogenetic distribution in 19 families (e.g. Equisetales, Polypodiales) and presence/absence in intrageneric taxa (e.g. *Pyrrhosia*) indicate multiple origins and secondary losses, especially during the last 50 million years of fern evolution. Differences between hydathodes of closely related fern

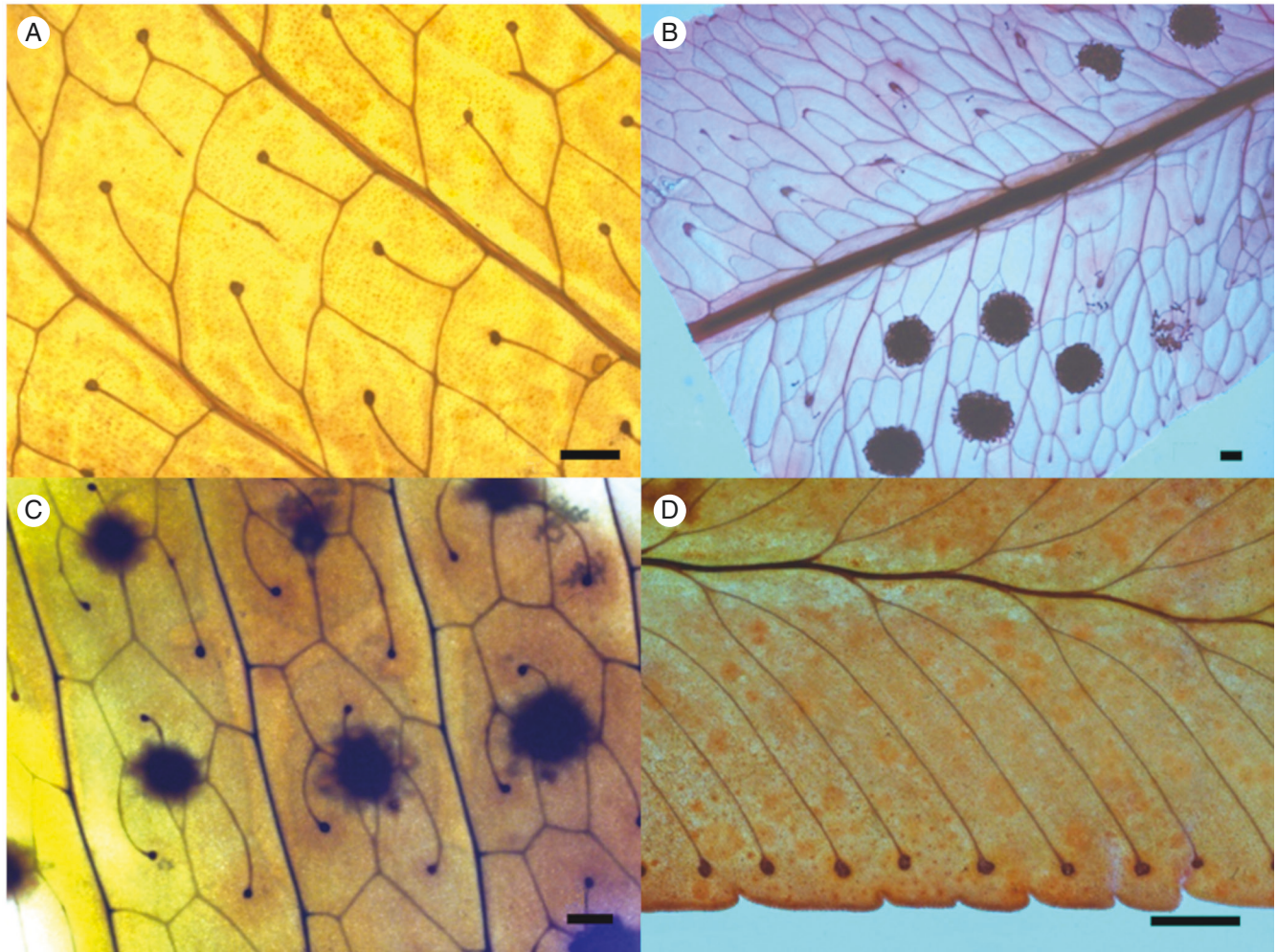


FIG. 4. Leaf venation of fern species with hydathodes on vein endings. (A–C) Reticulate venation with free vein endings. (A) *Campyloneurum brevifolium*, (B) *Phlebodium aureum*, (C) *Niphidium crassifolium*, (D) open venation with hydathodes: *Nephrolepis cordifolia*. Large dark spots in (B) and (C) represent sori. Scale bars = 1 mm.

species, such as the production or lack of salt residues (e.g. within *Aglaomorpha*; Janssen and Schneider, 2005), their presence in young but absence in mature leaves (e.g. *Pyrrosia nummularifolia*; Hovenkamp, 1986), and complete versatility within the same species (e.g. *Cryptogramma stelleri*; Morin, 1993), may indicate genetic mutations, changes during the ontogenetic development or functional changes triggered by environmental factors, respectively. However, the applied terminology for hydathodes was not used consistently in the past. Whereas for some authors a hydathode is simply a morpho-anatomical trait to refer to an enlarged or thickened vein end, others clearly relate it to its function in guttation. Concrete evidence for the activity of hydathodes is the appearance of salt residues after the guttation fluid has evaporated. Lime dots or cretaceous residues were reported in at least 100 of the hydathode-bearing species (Supplementary Data File S1). The lack of lime dots in most fern species may indicate that their hydathodes are inactive, that they release only minimum amounts of salts, or that these plants grow in mineral-poor soils with no excess of salts or at lower air humidity levels that do not promote guttation. However, none of these possible

explanations has been studied so far. It is also possible that in natural environments with frequent heavy rainfall salt residues could be washed away, and their presence would therefore be overlooked. However, our own observations suggest that this is unlikely, because in cultivated specimens in the greenhouse lime dots were not easily removed by spraying water.

Our stochastic character mapping supported multiple origins of hydathodes within ferns at the genus level. The number of reported gains and losses should be expected to increase once a complete data set for species-level analysis is available in the future. In any case, the observed structural and functional differences of hydathodes in plants have been considered as evidence for several independent origins. In vascular plants, hydathodes can differ in their locations on the leaves (apical, marginal, laminar on the upper or lower surface), consist of simple or stomata-like pores, can be connected or disconnected to leaf veins, possess loose or compact underlying epithem with or without intercellular spaces, and release guttation fluids of distinct chemical composition. Most hydathodes in angiosperm leaves are apical (in monocots, e.g. *Sparganium emersum*; Pedersen et al., 1997) or marginal (in dicots), with

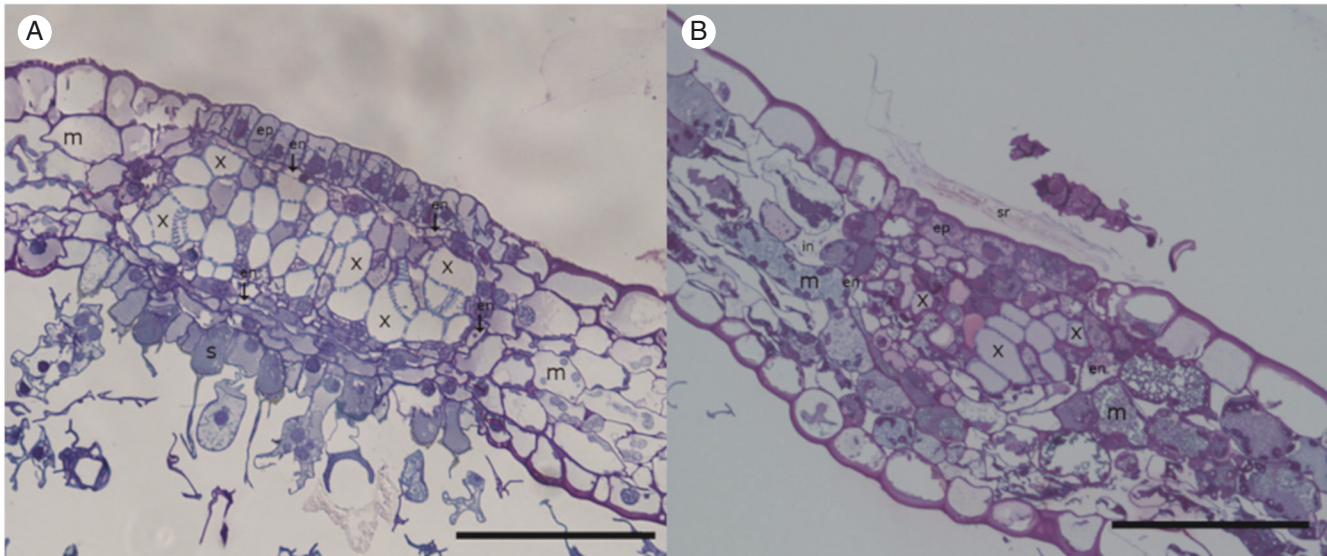


FIG. 5. Cross-sections of hydathodes of (A) *Phlebodium aureum* (with sorus on lower leaf surface) and (B) *Nephrolepis brownii* (with salt residues on top). en, endodermis-like tissue; ep, epidermis; in, intercellular space; m, mesophyll; s, sporogenous tissue; sr, salt residue; x, xylem. Scale bars = 100  $\mu\text{m}$ .

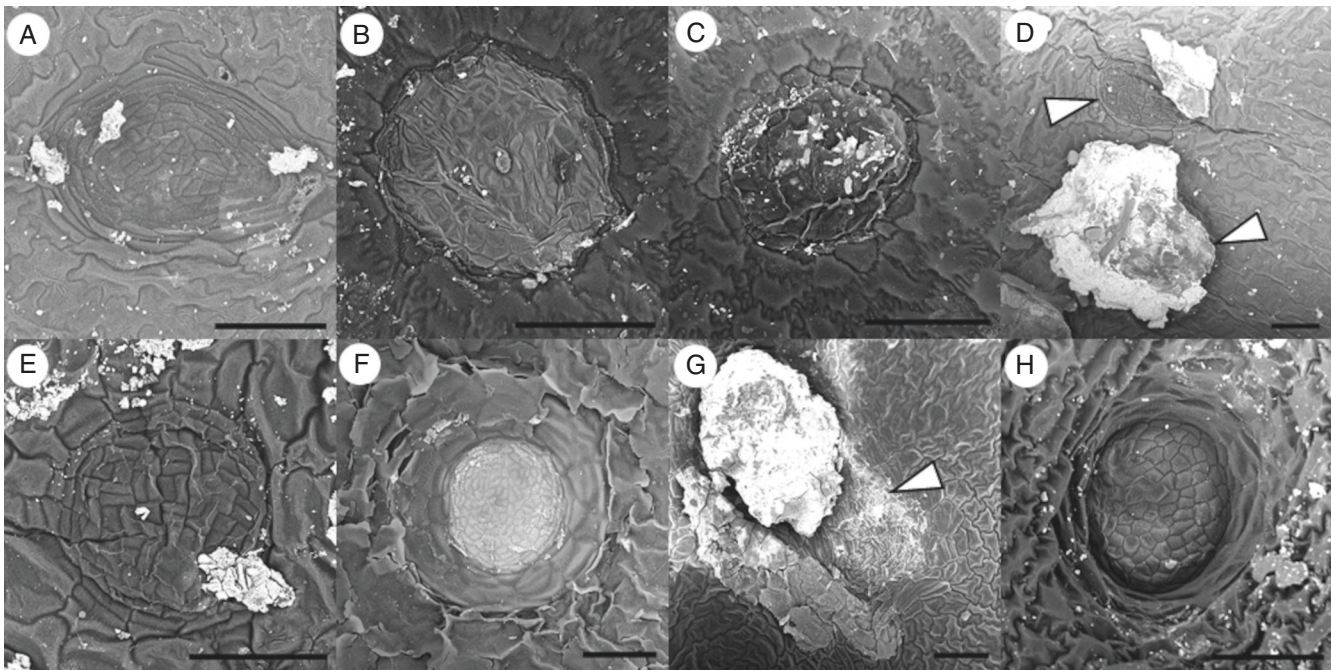


FIG. 6. SEM pictures of adaxial leaf surfaces with pore-free hydathodes after removal of salt residues (except D). Hydathode cells differ from surrounding epidermis cells by their small, compact size. (A) *Aglaomorpha pilosa*, (B) *Campyloneurum brevifolium*, (C) *Campyloneurum xalapense*, (D) *Nephrolepis brownii*, upper hydathode partially uncovered, lower hydathode covered by salt residue (see arrowheads), (E) *Nephrolepis cordifolia*, (F) *Niphidium crassifolium*, (G) *Phlebodium aureum*, lower part on the right uncovered (arrow), (H) *Pyrrosia polydactyla* with deeply sunken hydathode. Scale bars = 100  $\mu\text{m}$ .

few exceptions (e.g. Crassulaceae, Moraceae, Ranunculaceae, Urticaceae) that are laminar (Cerutti *et al.*, 2019), such as all the fern hydathodes observed here. In angiosperms, pore-bearing hydathodes (= water pores) may have evolved from stomata because they may share molecular markers (Cerutti *et al.*, 2019), and these pores in *Arabidopsis thaliana* also respond to abscisic acid but remain more open than stomata (Cerutti *et al.*, 2017). Because hydathodes of ferns lack pores and are located mainly on the upper leaf surface, it seems doubtful that they

have originated from stomata. However, hydathodes may have evolved into nectaries in both ferns (e.g. *Pleopeltis*, Koptur *et al.*, 1982) and angiosperms (Belin-Depoux, 1989). The latter author suggested that angiosperm hydathodes may have previously attracted ants. Cerutti *et al.* (2019) proposed the existence of a morphological continuum with intermediate structures and transitions between hydathodes, trichomes and nectaries, which may have evolved multiple times independently or from each other in both evolutionary directions. Observations of the fern



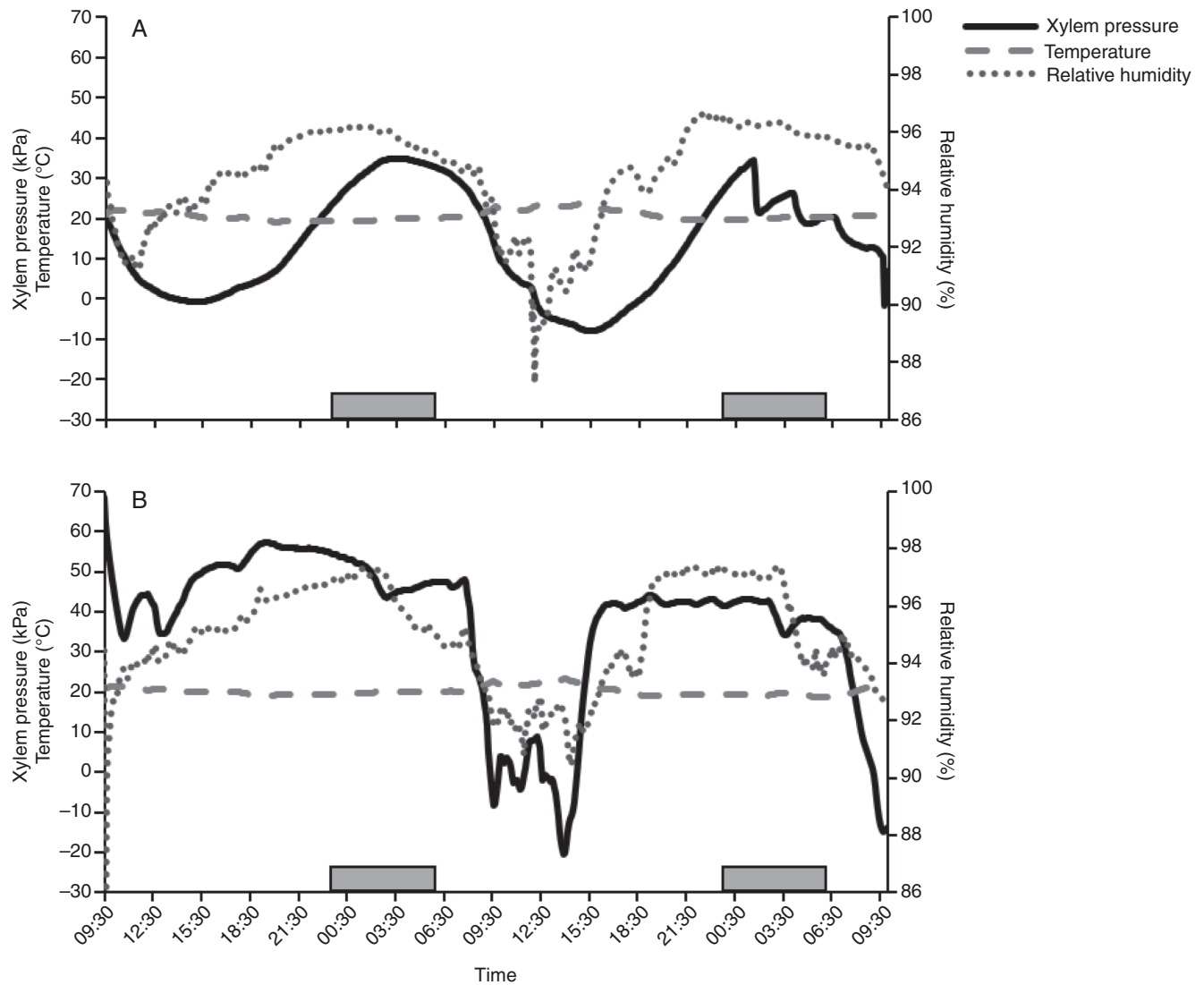


FIG. 7. Day–night cycle of xylem pressure of (A) *Phlebodium aureum* and (B) *Nephrolepis cordifolia* depending on temperature and relative air humidity. Grey rectangles indicate periods of guttation.

genus *Pleopeltis* support this idea, because most species in that genus bear both hydathodes and nectaries. In *Pleopeltis*, hydathodes occupy all the vein endings except for either the basal acroscopic vein, ending in pinnate-leaved species, or at the base of the lamina in simple-leaved species, which are occupied by nectaries. Detailed anatomical observations are required to determine homology or partial homology and to apply the correct terminology. The cretaceous hydathodes of our studied ferns were such intermediate structures because they were connected to the vascular system and worked passively depending on xylem pressure but lacked epidermal pores and intracellular spaces in the epithem, which are characteristics described as those of actively secreting salt glands (Fahn, 1979) and trichome hydathodes (Haberlandt, 1914; Heinrich, 1973; Ponzi and Pizzolongo, 1992; Evert, 2006). Consequently, in these fern species, hydathodes may have combined the two functions – the maintenance of a water flow to acquire essential nutrients driven by xylem pressure and the release of excess salts exuded

actively from epithem cells into the guttation fluid. For further conclusions, more detailed studies should investigate the possible morphological and functional differences between non-cretaceous and cretaceous hydathodes and the environmental conditions under which they operate.

#### Hydathode activity

Hydathodes were mainly active during the night when air humidity reaches the point of saturation. Because some species (e.g. *C. xalapense*) did not consistently develop lime dots under greenhouse conditions, the environmental conditions may also play an important role in the salt content of the guttation fluid. First, under greenhouse cultivation, plants are grown in often standardized soil substrates and watered with softer, mineral-poor water that may reduce the amount of excess salts. Second, in nature, species often undergo higher day/night temperature

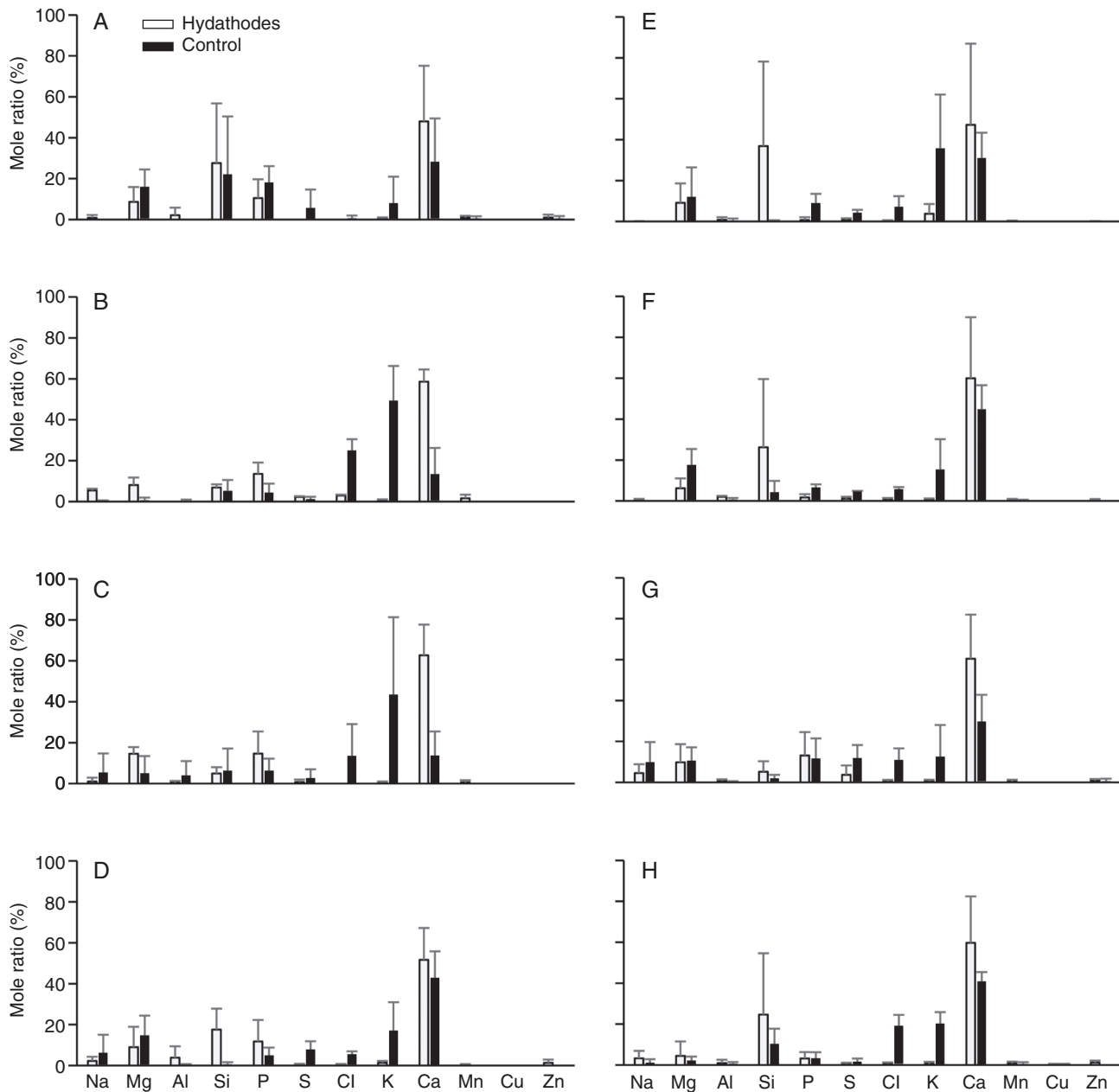


FIG. 8. Elemental composition of salt residues (means  $\pm$  s.d.) on hydathodes and epidermal surface as control (as indicated in key). (A) *Aglaomorpha pilosa*, (B) *Campyloneurum brevifolium*, (C) *Campyloneurum xalapense*, (D) *Nephrolepis brownii*, (E) *Nephrolepis cordifolia*, (F) *Niphidium crassifolium*, (G) *Phlebodium aureum*, (H) *Pyrrosia polydactyla*.

cycles, which may increase xylem pressure and the release of hydathode fluid at night and its evaporation during the day, leading to an increased deposition of salts. Because our results were limited to greenhouse experiments under relatively constant temperature and air humidity, the next step of research should focus on the effect of increasing differences in day/night temperatures on guttation. Some examples have already shown that guttation in aquatic plants decreased at lower temperatures (Pedersen, 1993).

In most species of this study, hydathode activity changed during leaf ontogeny. Hydathodes became active when leaves expanded to ~80–90 % of their final size, apparently when their

epithem became connected to the vascular tissue of the shoot but stopped exuding fluids a few weeks later. In many species, guttation is more intensive in young, expanding leaves or even restricted to the early stage of leaf development (Haberlandt, 1894; Pedersen, 1993; Singh 2016). It was suggested that hydathodes become inactive because the endodermis that surrounds the epithem is developing the Casparian strip, inhibiting apoplastic water transport from the epithem to the epidermis, and because the epidermis of mature leaves is sealed by additional layers of cutin or lignin (Priestley and Radcliffe, 1924; Kramer *et al.*, 1995). In contrast, we could observe neither a Casparian strip nor a cutinization of the endodermis-like tissue;

however, we observed a considerable variation of the time of active guttation among species. Leaves of some ferns (e.g. *P. aureum*) produced guttation droplets for up to 3 months. Perhaps guttation fluid is exuded at low rates for an even longer period, as indicated by the accumulation of larger amounts of salt residues in older leaves, but evaporates quickly without forming any conspicuous water droplets.

The most intriguing question, however, remains. What is the function of guttation when it is limited to developing leaves, and why does guttation stop in mature leaves? Guttation supposedly increases leaf transpiration at night to provide access to more dissolved nutrients in the xylem sap (Singh, 2016). Young, expanding leaves have low transpiration and photosynthetic rates but require larger amounts of nutrients for growth and development. In ferns, sporangial development occurs at the lower leaf surface, and often opposite to the hydathode-bearing vein end at the upper leaf surface (Fig. 5A), and increases the nutrient demand for the synthesis of DNA, storage proteins and lipids for developing spores. However, because sterile as well as fertile leaves have the same active hydathodes, even this appealing hypothesis does not withstand further consideration. Finally, Haberlandt (1894) observed that leaf halves that were treated with alcoholic mercuric chloride solutions suffered from hydathode poisoning and consequent flooding of the leaf mesophyll and lack of guttation, whereas hydathodes of untreated leaf halves were still exuding liquids. Feild *et al.* (2005) noted that blocking hydathodes with wax also caused mesophyll flooding and lowered CO<sub>2</sub> gas exchange and photosynthetic performance, but that even these outcomes did not severely affect the plants (Chen *et al.*, 2014). The former examples show that the hydathode functions may provide some physiological benefits but that these are not essential for plant survival.

#### Positive xylem pressure

We measured a maximum positive xylem pressure of 33.4 and 53.5 kPa in the two species *P. aureum* and *N. cordifolia*, respectively. These positive pressures are clearly sufficient to rehydrate the leaves of these species up to a height of 80–100 cm, because 100 kPa can raise water in a capillary to a height of 10 m, assuming no lumen or end wall resistance. Both species occur in the tropics at sun-exposed sites, where they can experience drought stress during the day, *P. aureum* as an epiphyte on tree branches and *N. cordifolia* as a terrestrial fern on hot, rocky substrates. For both species, it is imperative to recover quickly during rainfall and cloudy weather conditions when soil water becomes available and air humidity rises.

*Austroblechnum lehmannii*, a small understory fern with enlarged vein ends (Mickel and Smith, 2004) that might not be exposed to such frequent drought stress, only produces a positive xylem pressure of 12–17 kPa, still enough to hydrate its leaves of 30 cm length (Sperry, 1983). Clearly, the leaf length of the fern species seems to be related to maximum xylem pressure. Holmlund *et al.* (2020) discovered in two desiccation-tolerant ferns that *Pellaea andromedifolia* can produce a positive xylem pressure of >3 kPa and *Pentagramma triangularis* of 16 kPa, in both species sufficient to rehydrate their small leaves. On the other hand, Fisher *et al.* (1997) and Ewers *et al.* (1997)

measured a positive xylem pressure of 22 and 66 kPa on specimens of the climbing fern *Lygodium venustum* that reached up to 2 and 3 m height, respectively. Because leaves of this species can reach >10 m height (Mehltreter, 2006), it would be of special interest to investigate if they raise their positive xylem pressure with increasing leaf length. Although this species has no hydathodes and does not guttate, water is exuded after cutting the leaves at soil surface level as proof of xylem pressure (Fisher *et al.*, 1997). Consequently, we conclude that all currently available data suggest that measured fern species can produce a positive xylem pressure depending on their leaf sizes, and can rehydrate their leaves, but that this rehydration capacity is not necessarily related to the presence of hydathodes.

#### Salt residues of fern hydathodes

The elemental composition of salt residues provided evidence that hydathode function may differ significantly among species. Even when cultivated in the same greenhouse conditions and the same soil substrate as in our study, all species released mainly Ca but some of them also segregated considerable quantities of Si. In plants, Ca<sup>2+</sup> reinforces cell membranes and walls, and plays a role as a secondary messenger for environmental signals. It occurs in large concentrations within the tonoplast (1–2 M) but very low concentrations in the cytosol (~0.1 μM; Thor, 2019). Silicon occurs in all cell compartments in low concentrations because of its low solubility (<2 mM), is passively absorbed and plays a minor role in plants (Exley, 2015), except under stressful conditions such as nutrient limitation (Minden *et al.*, 2021). Consequently, larger amounts of Si in salt residues were surprising, because it represents only 2–3 % of the dry mass in horsetails (order Equisetales; Labun *et al.*, 2013). However, Si is absorbed and distributed in plants in the form of silicic acid Si(OH)<sub>4</sub> (Exley, 2015), and in many ferns it can be deposited as biogenic silica in palaeontologically diagnostic phytoliths, silicified parts of the plant that remain after decay or digestion of the plant (Sundue, 2009; Mazumdar, 2011). In some fern species, such as *Adiantum raddianum*, Si is specifically deposited in the walls of idioblastic, epidermal cells and stomatal guard cells (Leroux *et al.*, 2013). Because guttation is supposedly the only pathway for silicic acid to exit the plant (Exley, 2015), this would explain why silicon occurs in some amount in the salt residues of hydathode-bearing ferns, especially in those that do not deposit silica within the cell. Because all species studied have been cultivated in the same substrate, the differences in Si concentration in salt residues must be the consequence of metabolic differences among species. On the other hand, large amounts of Ca<sup>2+</sup> had to be expected in salt residues of the species studied because it is one of the most abundant nutrients in plants (Thor, 2019) and when it occurs in excess it is deposited as calcium oxalate crystals or exuded by the roots (Wu *et al.*, 2018).

The release of valuable elements such as P and Mg in the guttation fluid may indicate that hydathodes may not be able to avoid some leakage, whereas the presence of higher amounts of Al may indicate that hydathodes could provide some species with a functional mechanism to discard excess toxic minerals such as arsenic in *Pteris vittata* (Cantamessa *et al.*, 2016).

Because salt glands have been reported to exude  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$ ,  $\text{NO}_3^-$ ,  $\text{PO}_4^{3-}$  and  $\text{HCO}_3^-$  (Fahn, 1988) and these have an anatomy similar to that of the hydathodes of the studied ferns, we conclude that ferns possess hydathodes that functionally depend on xylem pressure, but may still actively alter the salt concentrations of guttation fluid to their required nutrient stoichiometry, retaining rare and valuable minerals but releasing excessive and toxic minerals such as Al. Future studies should investigate the variation of salt concentrations in xylem sap and of salt residues released by hydathodes within the same species depending on their concentrations and plant availability in the soil substrate.

### Conclusions

The ancestral character state reconstruction and diverse structure and presence of hydathodes in ~10 % of all known fern species suggest that they have originated multiple times and should fulfil an important function. However, only 10 % of the 1189 hydathode-bearing fern species are reported to produce salt residues consistently, which points to a possible regulatory function of leaf nutrient stoichiometry and active release of excessive compounds or waste products. It would be intriguing to gain a better understanding of the correlation between the composition of salt residues and soil cation content and other soil parameters (e.g. pH). Some open questions for future research are: What are the consequences of long-term hydathode blockage? Is guttation restricted to hydathode-bearing fern species that can produce positive xylem pressure? Under which environmental conditions do these fern species release guttation fluid? Further anatomical observations in a larger number of species are also needed to investigate the homology of hydathodes across ferns.

### SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. File S1: commented and referenced list of 1189 fern species with hydathodes. File S2: phylogeny of 273 fern genera with distribution of hydathode presence and type mapped. File S3: phylogeny of 199 fern genera with results of stochastic character mapping of hydathode presence/absence. File S4: time-lapse video clip of diurnal hydathode activity of *Phlebodium aureum* over 72 h.

### FUNDING

This work was supported by the Consejo Nacional de Ciencia y Tecnología (2019-000009-01EXTV-00147 to K.M.) and the Deutscher Akademischer Austauschdienst (57516480 to S.J.).

### ACKNOWLEDGEMENTS

The Botanical Gardens at Kew, Berlin (Nils Köster, Thomas Borowka, Tim Steege, Benjamin Grenz, Christel Schrader), Bochum (Thomas Stützel), Munich (Andreas Gröger, Harald Frank), Tübingen (Alexandra Kehl) and Ulm (Jutta

Siegmund-Jonitz, Monika Gschneidner, Peter Zindl) provided leaf material and living plants. We thank the staff at the Botanical Gardens in Ulm for their help in cultivating the living materials. The staff of the Central Facility for Electron Microscopy (Reinhard Weih, Andreas Ziegler) and Andrea Huppenberger helped with the preparation, analysis and photography of SEM samples, and Marco Tschapka kindly provided the camera equipment. We also thank Karolina Heyduk (Associate Editor) and two anonymous reviewers for their constructive feedback that helped improve the manuscript.

### LITERATURE CITED

- Belin-Depoux, M.** 1989. Des hydathodes aux nectaires foliaires chez les plantes tropicales. *Bulletin de la Société Botanique de France. Actualités Botaniques* **136**: 151–168. doi:10.1080/01811789.1989.10826969.
- Brodersen C, Jansen S, Choat B, Rico C, Pittermann J.** 2014. Cavitation resistance in seedless vascular plants: the structure and function of interconduit pit membranes. *Plant Physiology* **165**: 895–904. doi:10.1104/pp.113.226522.
- Brodrribb TJ, Bienaimé D, Marmottant P.** 2016. Revealing catastrophic failure of leaf networks under stress. *Proceedings of the National Academy of Sciences of the USA* **113**: 4865–4869. doi:10.1073/pnas.1522569113.
- Campany CE, Pittermann J, Baer A, et al.** 2021. Leaf water relations in epiphytic ferns are driven by drought avoidance rather than tolerance mechanisms. *Plant, Cell and Environment* **44**: 1741–1755. doi:10.1111/pce.14042.
- Cantamessa S, D'Agostino G, Berta G.** 2016. Hydathode structure and localization in *Pteris vittata* fronds and evidence for their involvement in arsenic leaching. *Plant Biosystems* **150**: 1208–1215. doi:10.1080/11263504.2015.1012135.
- Cerutti A, Jauneau A, Auriac M-C, et al.** 2017. Immunity at cauliflower hydathodes controls systemic infection by *Xanthomonas campestris* pv *campestris*. *Plant Physiology* **174**: 700–716. doi:10.1104/pp.16.01852.
- Cerutti A, Jauneau A, Laufs P, et al.** 2019. Mangroves in the leaves: anatomy, physiology, and immunity of epithelial hydathodes. *Annual Review of Phytopathology* **57**: 91–116. doi:10.1146/annurev-phyto-082718-100228.
- Chen YC, Lin TC, Martin CE.** 2014. Effects of guttation prevention on photosynthesis and transpiration in leaves of *Alchemilla mollis*. *Photosynthetica* **52**: 371–376. doi:10.1007/s11099-014-0040-y.
- Davidse G, Sousa M, Knapp S, eds.** 1995. *Flora Mesoamericana. Vol. 1: Psilotaceae-Salviniaceae*. Mexico, St Louis, London: Universidad Nacional Autónoma de México, Missouri Botanical Garden, and Natural History Museum.
- Davies PJ, ed.** 2010. *Plant hormones: biosynthesis, signal transduction, action!* 3rd edn. Dordrecht: Springer.
- Dieffenbach H, Kramer D, Lüttge U.** 1980. Release of guttation fluid from passive hydathodes of intact barley plants. I. Structural and cytological aspects. *Annals of Botany* **45**: 397–401. doi:10.1093/oxfordjournals.aob.a085837.
- Evert RF.** 2006. *Esau's plant anatomy*, 3rd edn. New Jersey: John Wiley and Sons, Inc.
- Ewers FW, Cochard H, Tyree MT.** 1997. A survey of root pressures in vines of a tropical lowland forest. *Oecologia* **110**: 191–196. doi:10.1007/s004420050149.
- Exley C.** 2015. A possible mechanism of biological silicification in plants. *Frontiers in Plant Science* **6**: 853. doi:10.3389/fpls.2015.00853.
- Fahn A.** 1979. *Secretory tissues in plants*. London: Academic Press.
- Fahn A.** 1987. Secretory tissues and factors influencing their development. *Phyton* **28**: 13–26.
- Fahn A.** 1988. Secretory tissues in vascular plants. *New Phytologist* **108**: 229–257. doi:10.1111/j.1469-8137.1988.tb04159.x.
- Feild TS, Sage TL, Czerniak C, Iles WJD.** 2005. Hydathodal leaf teeth of *Chloranthus japonicus* (Chloranthaceae) prevent guttation-induced flooding of the mesophyll. *Plant, Cell and Environment* **28**: 1179–1190. doi:10.1111/j.1365-3040.2005.01354.x.
- Fisher JB, Angeles G, Ewers F, López-Portillo J.** 1997. Survey of root pressure in tropical vines and woody species. *International Journal of Plant Sciences* **158**: 44–50.

- Gardiner W. 1883. On the physiological significance of water glands and nectaries. *Proceedings of the Cambridge Philosophical Society* 5: 35–50.
- Gasper AL, Almeida TE, Dittrich VAO, Smith AR, Salino A. 2016. A classification for Blechnaceae (Polypodiales: Polypodiopsida): new genera, resurrected names, and combinations. *Cladistics* 33: 429–446.
- Goatley JL, Lewis RW. 1966. Composition of guttation fluid from rye, wheat, and barley seedlings. *Plant Physiology* 41: 373–375. doi:10.1104/pp.41.3.373.
- Haberlandt G. 1894. Über Bau und Funktion der Hydathoden. *Berichte der Deutschen Botanischen Gesellschaft* 12: 367–378.
- Haberlandt G. 1914. *Physiological plant anatomy*. London: Macmillan.
- Heinrich G. 1973. Die Feinstruktur der Trichom-hydathoden von *Monarda fistulosa*. *Protoplasma* 77: 271–278. doi:10.1007/bf01276763.
- Hernández-Hernández V, Terrazas T, Mehtreter K, Angeles G. 2012. Studies of petiolar anatomy in ferns: structural diversity and systematic significance of the circumendodermal band. *Botanical Journal of Linnean Society* 169: 596–610. doi:10.1111/j.1095-8339.2012.01236.x.
- Holmlund HI, Davis SD, Ewers FW, et al. 2020. Positive root pressure is critical for whole-plant desiccation recovery in two species of terrestrial resurrection ferns. *Journal of Experimental Botany* 71: 1139–1150. doi:10.1093/jxb/erz472.
- Holtum RE. 1954. *A revised flora of Malaya, Vol. II: Ferns of Malaya*. Singapore: Government Printing Office.
- Hoshizaki BJ. 1991. An 'intergeneric' hybrid: *Aglaomorpha* × *Drynaria*. *American Fern Journal* 81: 37–43. doi:10.2307/1547518.
- Hovenkamp P. 1986. *A monograph of the fern genus Pyrrosia (Polypodiaceae)*. Leiden: E. J. Brill/Leiden University Press.
- Hovenkamp P. 1998. Polypodiaceae. In: *Flora Malesiana. Series II*. Leiden: Rijksherbarium. Vol. 3, 1–234.
- Hovenkamp P, Miyamoto F. 2005. A conspectus of the native and naturalized species of *Nephrolepis* (Nephrolepidaceae) in the world. *Blumea* 50: 279–322.
- Ingrouille M, Eddie B. 2006. *Plants: evolution and diversity*. Cambridge: Cambridge University Press.
- Janssen T, Schneider H. 2005. Exploring the evolution of humus collecting leaves in drynarioid ferns (Polypodiaceae, Polypodiidae) based on phylogenetic evidence. *Plant Systematics and Evolution* 252: 175–197. doi:10.1007/s00606-004-0264-6.
- John SP, Hasenstein H. 2017. The role of peltate scales in desiccation tolerance of *Pleopeltis polypodioides*. *Planta* 245: 207–220.
- Kessler M, Moguel VAL, Sundue M, Labiak PH. 2011. *Alansmia*, a new genus of grammitid ferns (Polypodiaceae) segregated from *Terpsichore*. *Brittonia* 63: 233–244.
- Klepsch M, Lange A, Angeles G, Mehtreter K, Jansen S. 2016. The hydraulic architecture of petioles and leaves in tropical fern species under different levels of canopy openness. *International Journal of Plant Sciences* 177: 209–216. doi:10.1086/684176.
- Koptur S, Smith AR, Baker I. 1982. Nectaries in some neotropical species of *Polypodium* (Polypodiaceae): preliminary observations and analyses. *Biotropica* 14: 108–113. doi:10.2307/2387739.
- Kramer KU, Green PS. eds. 1990. *Families and genera of vascular plants. Vol. 1: pteridophytes and gymnosperms*. Berlin: Springer.
- Kramer KU, Schneller JJ, Wollenweber E. 1995. *Farne und Farnerwandte: Morphologie, Systematik, Biologie*. Stuttgart: Thieme.
- Labun P, Grulova D, Salamon I, Šeršeň F. 2013. Calculating the silicon in horsetail (*Equisetum arvense* L.) during the vegetation season. *Food and Nutrition Sciences* 4: 510–514.
- Lagoria M, Avila G, Neira DA, et al. 2018. Morphoanatomical and histochemical characteristics of the epiphytic fern *Pleopeltis macrocarpa* (Polypodiaceae). *Brazilian Journal of Botany* 41: 739–750. doi:10.1007/s40415-018-0474-8.
- León B. 1993. *A taxonomic revision of the fern genus Campyloneurum (Polypodiaceae)*. PhD Thesis, University of Aarhus, Denmark.
- Leroux O, Leroux F, Mastroberti AA, et al. 2013. Heterogeneity of silica and glycan-epitope distribution in epidermal idioblast cell walls in *Adiantum raddianum* laminae. *Planta* 237: 1453–1464. doi:10.1007/s00425-013-1856-6.
- Limm EB, Simonin KA, Bothman AG, Dawson TE. 2009. Foliar water uptake: a common water acquisition strategy for plants of the redwood forest. *Oecologia* 161: 449–459. doi:10.1007/s00442-009-1400-3.
- Mahley JN, Pittermann L, Rowe N, et al. 2018. Geometry, allometry and biomechanics of fern leaf petioles: their significance for the evolution of functional and ecological diversity within the Pteridaceae. *Frontiers in Plant Science* 9: e197. doi:10.3389/fpls.2018.00197.
- Martin CE, van Willert DJ. 2000. Leaf epidermal hydathodes and the ecophysiological consequences of foliar water uptake in species of *Crassula* from the Namib Desert in Southern Africa. *Plant Biology* 2: 229–242.
- Mazumdar J. 2011. Phytoliths of pteridophytes. *South African Journal of Botany* 77: 10–19. doi:10.1016/j.sajb.2010.07.020.
- McCarthy PM. ed. 1998. *Flora of Australia. Vol. 48: ferns, gymnosperms and allied groups*. Melbourne: ABR/CSIRO Australia.
- Mehrtreter K. 2006. Leaf phenology of the climbing fern *Lygodium venustum* in a semideciduous lowland forest on the Gulf of México. *American Fern Journal* 96: 21–30. doi:10.1640/0002-8444(2006)96[21:lpotcf]2.0.co;2.
- Mehrtreter K, Walker LR, Sharpe JM. eds. 2010. *Fern ecology*. Cambridge: Cambridge University Press. doi:10.1017/CBO9780511844898.
- Mehrtreter K, Tenhaken R, Jansen S. 2022. Nectaries in ferns: their taxonomic distribution, structure, function, and sugar composition. *American Journal of Botany* 109: 46–57. doi:10.1002/ajb2.1781.
- Mickel JT, Smith AR. 2004. *The pteridophytes of Mexico*. New York: The New York Botanical Garden Press.
- Minden V, Schaller J, Venterink HO. 2021. Plants increase silicon content as a response to nitrogen or phosphorus limitation: a case study with *Holcus lanatus*. *Plant and Soil* 462: 95–108. doi:10.1007/s11104-020-04667-1.
- Moran RC, Labiak PH. 2016. Phylogeny and character evolution of the Neotropical fern genus *Stigmatopteris* (Dryopteridaceae). *Brittonia* 68: 476–488. doi:10.1007/s12228-016-9437-1.
- Morin NR, ed. 1993. *Flora of North America. Vol. 2: pteridophytes and gymnosperms*. New York, Oxford University Press.
- Paradis E, Schliep K. 2019. Ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics* 35: 526–528. doi:10.1093/bioinformatics/bty633.
- Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* 20: 289–290. doi:10.1093/bioinformatics/btg412.
- Pedersen O. 1993. Long-distance water transport in aquatic plants. *Plant Physiology* 103: 1369–1375. doi:10.1104/pp.103.4.1369.
- Pedersen O, Jørgensen L, Sand-Jensen K. 1997. Through-flow of water in leaves of submerged plant is influenced by the apical opening. *Planta* 202: 43–50.
- Pittermann J, Limm E, Rico C, Christman MA. 2011. Structure-function constraints of tracheid-based xylem: a comparison of conifers and ferns. *New Phytologist* 192: 449–461. doi:10.1111/j.1469-8137.2011.03817.x.
- Ponzi R, Pizzolongo P. 1992. Structure and function of *Rhinanthus minor* L. trichome hydathode. *Phytomorphology* 42: 1–6.
- PPG I. 2016. A community-derived classification for extant lycophytes and ferns. *Journal of Systematics and Evolution* 54: 563–603.
- Priestley JH, Radcliffe FM. 1924. A study of the endodermis in the Filicinae. *New Phytologist* 23: 161–193. doi:10.1111/j.1469-8137.1924.tb06632.x.
- R Core Team. 2020. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. www.R-project.org.
- Ranker TA, Smith AR, Parris BS, et al. 2004. Phylogeny and evolution of grammitid ferns (Grammitidaceae): a case of rampant morphological homoplasy. *Taxon* 53: 415–428. doi:10.2307/4135619.
- Revell LJ. 2012. Phytools: an R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution* 3: 217–223.
- Schenk HJ, Jansen S, Hölltä T. 2020. Positive pressure in xylem and its role in hydraulic function. *New Phytologist* 230: 25–47. doi:10.1111/nph.17085.
- Schneider H, Schuettelpelz E, Pryer KM, Cranfill R, Magallon S, Lupia R. 2004. Ferns diversified in the shadow of angiosperms. *Nature* 428: 553–557. doi:10.1038/nature02361.
- Singh S. 2016. Guttation: mechanism, momentum, and modulation. *Botanical Review* 82: 149–182. doi:10.1007/s12229-016-9165-y.
- Smith AR. 1995. Grammitidaceae. In: Davidse G, Sousa MS, Knapp S. eds. *Flora Mesoamericana. Vol. 1. México, D.F.: Universidad Nacional Autónoma de México*, 366–393.
- Smith AR, Kessler M. 2018. Prodrómus of a fern flora for Bolivia. XXXVII. Nephrolepidaceae. *Phytotaxa* 334: 135–140. doi:10.11646/phytotaxa.334.2.3.
- de la Sota ER. 1966. Revisión de las especies americanas del grupo "Polypodium squamatum" L. (Polypodiaceae s. str.). *Revista del Museo de La Plata, Sección Botánica* 10: 69–186.

- de la Sota ER, Cassa de Pazos LA, Ponce MA. 2000. Grammitidaceae (Pteridophyta) de Argentina y Chile. *Darwiniana* **38**: 299–306.
- Sperry JS. 1983. Observations on the structure and function of hydathodes in *Blechnum lehmannii*. *American Fern Journal* **73**: 65–72. doi:10.2307/1546852.
- Sundue MA. 2009. Silica bodies and their systematic implications in Pteridaceae (Pteridophyta). *Botanical Journal of the Linnean Society* **161**: 422–435.
- Sundue MA. 2010a. A morphological cladistic analysis of *Terpsichore* (Polypodiaceae). *Systematic Botany* **35**: 716–729. doi:10.1600/036364410x539808.
- Sundue MA. 2010b. A monograph of *Ascogrammitis*, a new genus of grammitid ferns (Polypodiaceae). *Brittonia* **62**: 357–399. doi:10.1007/s12228-009-9108-6.
- Sundue MA. 2013. *Mycopteris*, a new neotropical genus of grammitid ferns (Polypodiaceae). *Brittonia* **66**: 174–185. doi:10.1007/s12228-013-9322-0.
- Sundue MA, Islam MB, Ranker TA. 2010a. Systematics of grammitid ferns (Polypodiaceae): using morphology and plastid sequence data to resolve the circumscriptions of *Melpomene* and the polyphyletic genera *Lellingeria* and *Terpsichore*. *Systematic Botany* **35**: 701–715. doi:10.1600/036364410x539790.
- Sundue MA, Rouhan G, Moran RC. 2010b. *Megalastrum* (Dryopteridaceae) of the circumaustral region: Chile, Argentina, and southern islands of the Atlantic, Pacific, and Indian Oceans. *Systematic Botany* **35**: 461–475.
- Sundue MA, Labiak PH, Mostacero J, Smith AR. 2012. *Galactodenia*, a new genus of grammitid ferns segregated from *Terpsichore* (Polypodiaceae). *Systematic Botany* **37**: 339–346.
- Testo W, Sundue M. 2016. A 4000-species dataset provides new insight into the evolution of ferns. *Molecular Phylogenetics and Evolution* **105**: 200–211. doi: 10.1016/j.ympev.2016.09.003.
- Thor K. 2019. Calcium—nutrient and messenger. *Frontiers in Plant Science* **10**: 440. doi:10.3389/fpls.2019.00440.
- Tizro P, Choic C, Khanlou N. 2019. Sample preparation for transmission electron microscopy. In: Yong WH, ed. *Biobanking: methods in molecular biology*. Vol. 1897. New York: Humana Press, 417–424.
- Vasco A, Thadeo M, Conover M, Daly DC. 2014. Preparation of samples for leaf architecture studies, a method for mounting cleared leaves. *Applications in Plant Sciences* **2**: 1400038. doi:10.3732/apps.1400038.
- Watkins JE, Holbrook NM, Zwieniecki MA. 2010. Hydraulic properties of fern sporophytes: consequences for ecological and evolutionary diversification. *American Journal of Botany* **97**: 2007–2019. doi:10.3732/ajb.1000124.
- Wild A, Schmitt V. 2012. *Biochemische und physiologische Versuche mit Pflanzen*. Berlin: Springer Spektrum.
- Wu F, Xu F, Ma X, Luo W, Luo L, Wong MH. 2018. Do arsenate reductase activities and oxalate exudation contribute to variations of arsenic accumulation in populations of *Pteris vittata*? *Journal of Soils and Sediments* **18**: 3177–3185. doi: 10.1007/s11368-018-1987-2.
- Zhengyi W, Raven P. 2013. *Flora of China*. Vol. 2-3. *Illustrations. Lycopodiaceae through Polypodiaceae*. St Louis: Missouri Botanical Garden Press.