The evolution of aluminum accumulation in ferns and lycophytes[1]

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PREMISE OF THE STUDY: This paper investigates the occurrence and evolution of aluminum (Al) accumulation within ferns and lycophytes, which is characterized by Al concentrations above 1000 mg·kg^{-1} in aboveground plant tissues. We hypothesize that this feature is more common in ferns than in angiosperms, and potentially correlated with growth form and other chemical elements.

METHODS: Aluminum concentrations were obtained from novel analyses and literature for a total of 354 specimens and 307 species. Moreover, a semi-quantitative aluminon test was applied for a subset of 105 species and validated against exact Al measurements.

KEY RESULTS: Molecular phylogenetic analyses showed that the major Al-accumulating groups were primarily found in the Gleicheniales and Cyatheales, and largely absent in the Polypodiales. At the species and generic level, Al accumulation was typically either absent or present, and mixed results within a single species and genus were limited to less than 30% of the species and genera tested. Epiphytic ferns had significantly lower Al levels than terrestrial ferns, although this finding was not significant after phylogenetic correction. In addition, a significant, positive correlation was found between Al and iron, while Al was negatively correlated with phosphorus and potassium concentrations.

CONCLUSIONS: Aluminum accumulation is most common outside of the Polypodiales and occurs in 38% of the species studied, indicating that this trait is indeed common within subtropical and tropical ferns, a finding that could be in line with their role as pioneer species on landslides and soils with high levels of soluble Al.

  KEY WORDS accumulation; aluminum; phylogenetic distribution; aluminon; elemental analysis

Aluminum (Al) is one of the most abundant elements of Earth's crust, where it is mostly bound in form of aluminosilicates. At a low soil pH (i.e., <5.5), however, phytotoxic trivalent Al^{3+} ions and related hydroxides (Al(OH)$_2^+$, Al(OH)$^{2+}$) are solubilized, enter the aqueous soil solution, and can be absorbed by plant roots. It has been shown that immediate responses of plants that are sensitive to Al include the inhibition of root growth and subsequent impairment of nutrient uptake (Kochian et al., 2004; Brunner and Sperisen, 2013; Grevenstuk and Romano, 2013; Leitenmaier and Küpper,

2013; Kopittke et al., 2015). Besides direct effects of Al toxicity on many crop plants (e.g., wheat, maize, rice, common bean), Al tolerance and exclusion mechanisms play a major role in plant growth and species distribution on acidic soils (von Uexküll and Mutert, 1995; Yang et al., 2013). Plant species that tolerate high levels of Al ions have developed various strategies to reduce Al stress on acidic soils, either by avoiding uptake into the plant body via secretion of chelators like organic acids into the rhizosphere, or by detoxifying Al within the plant body, for instance in apoplastic tissue in leaves and bark, where high levels of Al can be found (Jansen et al., 2002; Watanabe and Osaki, 2002; Kochian et al., 2004; Poschenrieder et al., 2008; Grevenstuk and Romano, 2013).

Depending on the level of Al resistance, most flowering plants tolerate a wide range of exchangeable Al concentrations via exclusion mechanisms to avoid Al toxicity (Matsumoto, 2000). Typical Al levels in these Al excluders are around 200 mg·kg^{-1}. A small number of angiosperms, including probably 5% of all eudicots and less than 1% of monocots, are Al accumulators, which accumulate by definition more than 1000 mg·kg^{-1} Al in their aboveground tissues (Chenery, 1948,1949a, b). Species belonging to this group, such

as the tea shrub (*Camellia sinensis*), various Melastomataceae and Rubiaceae, detoxify Al symplastically with organic compounds in their cell vacuoles, or apoplastically by crosslinking it to cell walls (Ma, 2000).

Apart from seed plants, ferns and lycophytes are the largest groups of vascular plants with about 10,578 and 1338 plant species, respectively (Pteridophyte Phylogeny Group I, 2016). Lycophytes are a sister group of the Euphyllophytes, which include ferns and seed plants. Lycophytes and ferns have played an important role in early land plant evolution (Pryer et al., 2001). Although recent phylogenetic studies have contributed substantially to our understanding of fern and lycophyte evolution, there are still conflicting and unresolved phylogenetic problems (Pryer et al., 2004; Lehtonen, 2011; Rothfels et al., 2013, 2015; Burnard et al., 2016; Field et al., 2016; Testo and Sundue, 2016). Current evolutionary insights suggest that ferns are no longer considered as "evolutionary holdovers" from the late Palaeozoic and early Mesozoic eras (Crane et al., 1995). More than 80% of the Polypodiales diversified during the Cretaceous period in ecological habitats created by angiosperms (Schneider et al., 2004), and have radiated into epiphytic and terrestrial habitats with a broad variation in light levels (Schuettpelz and Pryer, 2009; Watkins and Cardelus, 2012; Sundue et al., 2015; Testo and Sundue, 2016). Ferns show a wide geographical distribution and have adapted remarkably well to a wide range of extreme environments, including both tropical and cold temperate climates, alpine and lowland regions, as well as aquatic and xeric conditions (Mehltreter, 2008). Several fern species are pioneer plants of sites disturbed by natural events such as landslides, volcanic eruptions, and hurricanes, or human activities such as deforestation and fires (Spicer et al., 1985; Walker, 1994; Ranker et al., 2000; Walker and Sharpe, 2010).

Despite the significance of ferns and lycophytes in the evolution of land plants and their functional adaptations to a wide range of environments, we know little about how these vascular plants deal with the uptake or exclusion of minerals (either essential or toxic). Recently, the unique characteristics of a few fern species have been used for the phytoremediation of soils contaminated with arsenic (As) (Salido et al., 2003). Seven fern species in the genus *Pteris* and two species of *Pityrogramma* (Pteridaceae) have been found to accumulate As, suggesting that As accumulation is limited to a small number of species and represents a rather novel trait (Meharg, 2002; Zhao et al., 2002; Kertulis-Tartar et al., 2006). Since then, many papers have explored the physiological and functional mechanisms as well as the genetics of As accumulation and detoxification in the Chinese brake fern (*Pteris vittata*) and related species (Francesconi et al., 2002; Fayiga and Ma, 2005; Luongo and Ma, 2005; Kertulis-Tartar et al., 2006; Srivastava et al., 2006; Wei and Chen, 2006; Rathinasabapathi et al., 2007). There are various other records of heavy metal accumulation in ferns, including accumulation of cadmium (Cd), zinc (Zn), copper (Cu), and lead (Pb) in species such as *Athyrium yokoscense*, *Osmundastrum cinnamomeum*, and *Polypodium cambricum* (Francis and Petersen, 1983; Nishizono et al., 1987; Van et al., 2006; Roccotiello et al., 2015).

Aluminum accumulation has been reported in various fern families, accounting for approximately 43% of the fern species tested (Chenery, 1949a), which is considerably higher than the estimated number of Al accumulating angiosperm species (Jansen et al., 2002). Most of the earlier records of Al accumulating ferns, however, are based on the semiquantitative aluminon test (Chenery, 1949a; Webb, 1954; Moomaw et al., 1959), which has been validated for

10 species of Rubiaceae (Jansen et al., 2003). Chenery (1949b) concluded that Al accumulation was characteristic of eusporangiate ferns. Although Webb (Webb, 1954) confirmed this hypothesis, he also suggested that accumulation may have evolved more than once across the fern phylogeny. Because of recent progress in our phylogenetic understanding of ferns, we are now in a much better position to evaluate evolutionary trends in this group of vascular plants (Smith et al., 2006). Because the soil is considered to be the major pathway for Al to enter the plant, the substrate on which the plant is growing should also have an affect on Al uptake (Ma and Hiradate, 2000; Rengel, 2004; Brunner and Sperisen, 2013). Therefore, we hypothesize that most Al accumulating ferns are terrestrial rather than epiphytic or epipetric species. Physiological and biochemical pathways of Al within the plant are still poorly understood, and it is unclear whether other elements, such as calcium (Ca) or silicon (Si), influence the uptake of Al or if the metal may prevent toxicity symptoms of other elements like iron (Fe), as reported for *Camellia sinensis* (Cronan and Grigal, 1995; Watanabe et al., 1997; Britez et al., 2002; Rengel and Zhang, 2003; Hajiboland et al., 2013). Correlations between different chemical elements might therefore be an indicator for potential uptake, transport, and detoxification mechanisms within these plants, which has not yet been investigated in detail within ferns.

In this paper we investigate three things: (1) the evolution of Al accumulation across a wide range of ferns and lycophytes, including terrestrial and epiphytic ferns; (2) the possible correlations between Al and other chemical elements such as calcium (Ca), phosphorus (P), magnesium (Mg), iron (Fe), sodium (Na), and potassium (K); and (3) a comparison of Chenery's semiquantitative data based on the aluminon test with exact Al concentrations in leaves from a large number of species. Based on findings from Chenery (1949a), we expect that Al accumulation is rather common and mainly occurs in tropical, terrestrial fern, and lycophyte species growing on acidic soils (pH <5.5). Because of the lack of soil data, however, we are unable to investigate the effect of soil chemistry on Al accumulation in this study. Nevertheless, our results provide a general framework for studies on Al uptake, translocation, speciation, future distribution patterns of fern species, and Al detoxification in ferns. It may also help us with the identification of genes implicated in nutrient uptake in ferns.

MATERIALS AND METHODS

Plant material—Small leaf fragments from herbarium material were collected at the herbarium of the Royal Botanic Gardens, Kew (London, United Kingdom). A total number of 164 samples, representing 149 species, 110 genera, and 38 families were obtained. Chenery's aluminon test was applied to all herbarium samples, while quantitative analytic measurements based on inductively coupled plasma mass spectrometry (ICP-MS) were conducted on 113 samples (105 species) where sufficient leaf material was available. Additional quantitative measurements were performed on samples collected in Mexico and Japan, resulting in an overall data set of 187 specimens, representing 171 species from 107 genera and 45 families. Voucher specimens for all samples can be found at the TRY Plant Trait Database (https://www.try-db.org/TryWeb/Home.php; Kattge et al., 2011).

In addition, Al concentrations were compiled from literature for 167 specimens, 161 species, 64 genera, and 28 families (Chenery,

1949b; Osaki et al., 1998, 2003; Watanabe et al., 2007; Olivares et al., 2009).

Our overall, combined data set based on our own measurements and literature included 307 species (354 specimens, 49 families and 129 genera). We followed the PPG I family classification (Pteridophyte Phylogeny Group I, 2016). The habit of each species (terrestrial or epiphytic) was reported based on information from herbarium labels or literature. Soil data (soil pH and soil nutrient concentrations) were limited to <20 species (Osaki et al., 2003; Olivares et al., 2009) and could therefore not be considered in our analyses. A taxonomic overview of the number of species studied for each order based on PPG I (Pteridophyte Phylogeny Group I, 2016), and the percentage of the total diversity investigated is shown in Table 1. We had data from all 14 orders recognized by Pteridophyte Phylogeny Group I (2016), but we sampled less than 8% of the species in most of them. For only five orders of the 14 orders did we have data from more than 10% of the species.

To investigate the potential relationship between climate and Al accumulation, we assigned all specimens into the following climatic zones based on geographical location and distribution patterns: (1) exclusively tropical (146 specimens), (2) tropical to subtropical (110 specimens), (3) exclusively subtropical (25 specimens), (4) subtropical to temperate (41 specimens), (5) exclusively temperate (11 specimens), and (6) cosmopolitan (20 specimens). We used the climatic classification system following Köppen (1936). Because of the relatively low number of samples from temperate areas and a continuum between the above-mentioned climate categories, we analyzed the frequency of Al accumulators for only three arbitrary climate groups: (1) tropical to subtropical (including the categories 1, 2, and 3 listed above; $n = 281$ specimens); (2) subtropical to temperate (including categories 4 and 5; $n = 52$ specimens); and (3) cosmopolitan (i.e., category 6; $n = 20$ specimens). Therefore, all species were assigned to a single group and there was no overlap in species between the three climate groups.

Aluminum measurements—Aluminum accumulation was determined with the aluminon test, as described by Chenery (1948) and

previously applied by Jansen et al. (2000; 2004; 2002). The aluminon tests were categorized into four arbitrary categories based on their color: 0 (negative, yellow-to-dark brown color); 1 (weak Al accumulation, pinkish color); 2 (positive, pinkish-to-red color); and 3 (strongly positive, solution dark red-to-crimson).

Exact Al concentrations were measured using ICP-MS at Hokkaido University. Briefly, the samples were ground with nonmetallic scissors and dried in an oven at 80°C for 72 h. Dried samples were then digested with HNO_3 (EL grade, Kanto Chemical Co., Inc., Tokyo, Japan) and H_2O_2 (semiconductor grade, Santoku Chemical, Tokyo, Japan) in a DigiPREP apparatus (SCP Science, Quebec City, Canada). The digested solution was then used to take measurements with an ICP-MS. Because of the limited amount of leaf material, we were unable to apply this protocol to 44 species that were also used for the aluminon test. Additional measurements were conducted in Mexico using atomic absorption spectrometry (AAS) for 23 species (97 specimens). The Al concentrations in the literature data were measured with AAS (Osaki et al., 1998, 2003; Olivares et al., 2009), neutron-activation analysis (Watanabe et al., 2007), and determined photometrically (Chenery, 1949b).

Besides Al, elemental quantifications were determined for Ca, Fe, K, Mg, Na, and P using ICP-MS and AAS. We also measured the following metals using ICP-MS: arsenic (As), cadmium (Cd), copper (Cu), manganese (Mn), nickel (Ni), and zinc (Zn). The threshold values for defining accumulations of these metals were $1\ mg \cdot kg^{-1}$ for Cd; $1000\ mg \cdot kg^{-1}$ for As, Cu, and Ni; and $10\,000\ mg \cdot kg^{-1}$ for Mn and Zn (Baker and Brooks, 1989; Ma et al., 2001). Arsenic and Cd concentrations were measured for 146 specimens, while Cu, Mn, Ni, and Zn were measured for a total of 176 specimens.

The compiled data set and corresponding reference list is accessible via the TRY Plant Trait Database (https://www.try-db.org/TryWeb/Home.php; Kattge et al., 2011).

Phylogenetic analysis—We reconstructed the phylogenetic history of Al accumulation in ferns by mapping our data onto the 4000-taxon time-calibrated phylogeny of Testo and Sundue (2016). That tree was the maximum clade credibility chronogram generated from six chloroplast markers (*atpB*, *rbcL*, *rps4* gene, *rps4-trnS* IGS, *trnL* gene, *trnL-trnF* IGS) for 3973 fern species (excluding 34 outgroup taxa). It was calibrated with 26 vascular plant fossils. To conduct our analyses, we removed taxa with missing Al accumulation data from the chronogram, resulting in a final pruned tree of 175 species. If more than one specimen was available for a species, the mean Al value was calculated. Ancestral character states of log-transformed Al accumulation data were estimated using Maximum Likelihood with the *contMap* function in the 'phytools' package (Revell, 2012, 2013) in R (R Development Core Team, 2010).

We assessed the phylogenetic signal of Al accumulation in ferns using Pagel's λ and Blomberg's *K* (Pagel, 1999; Freckleton et al., 2002; Blomberg et al., 2003). Pagel's λ is a scaling factor where λ = 1 indicates trait evolution following a Brownian motion model, and λ = 0 indicates a lack of phylogenetic signal in the trait. Blomberg's *K* expresses the strength of phylogenetic signal as the ratio of observed signal in a trait on the phylogeny to that of the signal under Brownian motion. A value of *K* = 1 indicates evolution as expected under Brownian motion, *K* > 1 indicates that close relatives are more similar than expected, and *K* < 1 indicates high lability, at least at the tips of the tree. Randomization was used to provide a null hypothesis. These were implemented in R using the *phylosig* function in 'phytools' (Revell, 2012, 2013).

TABLE 1. Taxonomic overview of the number of fern and lycophyte species studied at the ordinal level, including a total of 307 species, 129 genera, and 49 families. The total number of species per order was based on the Pteridophyte Phylogeny Group I (PPG I), which was used to determine the percentage of species studied for each order. The number of aluminum accumulating species is based on the $1000\ mg \cdot kg^{-1}$ threshold for aboveground tissues.

Order	Total number of species (PPG I)	Number of species studied	Percentage of species studied	Number of Al accumulating species
Lycopodiales	388	10	2.6	9
Isoëtales	250	3	1.2	1
Selaginellales	700	3	0.4	1
Equisetales	15	2	13.3	0
Psilotales	17	2	11.8	0
Ophioglossales	112	4	3.6	1
Marattiales	111	21	18.9	18
Osmundales	18	6	33.3	0
Hymenophyllales	434	3	0.7	1
Gleicheniales	172	26	15.1	19
Schizaeales	190	5	2.6	0
Salviniales	82	4	4.9	2
Cyatheales	713	51	7.2	38
Polypodiales	8714	167	1.9	26

Statistical analyses—All statistical analyses were performed using R (R Development Core Team, 2010). We tested for a correlation between plant habitat (epiphytic vs. terrestrial) and Al accumulation values using the method of Ives and Garland (2010), implemented in the R package 'phylolm' (Ho and Ané, 2013). We employed the pruned chronogram of 175 species to examine if our independent trait (Al accumulation) predicts values of a binary dependent trait (habit) while accounting for the non-independence of the data points because of their phylogenetic relationships. Data were analyzed in R using functions from the 'ape' (Paradis et al., 2004), 'Geiger' (Harmon et al., 2008), and 'phytools' (Revell, 2012, 2013) packages, and the data were first tested for normal distribution using the Shapiro-Wilk-Test. Because none of the data followed a normal distribution, the nonparametric pairwise Wilcoxon rank-sum test with Bonferroni correction was used to compare differences between group means. Furthermore, nonparametric Spearman-correlation was applied to check for correlations between Al and various nutrients (including Ca, Fe, Mg, Na, P, K). In these correlation analyses, Al accumulating and nonaccumulating species were analyzed both separately and combined.

RESULTS

Evolution of Al accumulation—The mean Al level was 2592 mg·kg^{-1} ± 4567 (SD; n = 354 specimens). Based on the 1000 mg·kg^{-1} threshold, Al accumulation was present in 37.8% of the fern species studied, corresponding to 116 out of 307 species (Fig. 1, 2). In general, Al accumulation was either present or absent at the species level: only values above or below 1000 mg·kg^{-1} were found for 28 out of 35 species with at least two independent samples tested (Table 2). Therefore, intraspecific variation with Al accumulating and nonaccumulating specimens was limited to 20% of the species for which more than one sample was tested, including *Alsophila spinulosa*, *Angiopteris evecta*, *Asplenium cordatum*, *Dipteris conjugata*, *Equisetum hyemale*, *Nephrolepis cordifolia*, and *Odontosoria chinensis*). These species were not included in calculating the percentage of accumulating species.

At the genus level, our data set included 48 genera for which we had more than one species analyzed. Out of these 48 genera, 8 had exclusively Al-accumulators, 26 had only nonaccumulators, and 14 included both accumulators and nonaccumulators. Therefore, Al accumulation was consistently present or absent at the genus level in 71% of all 48 genera, with a mixed occurrence in 29% of the genera studied.

The mean Al concentration was 6251 ± 5705 mg·kg^{-1} and 309 ± 245 mg·kg^{-1} for Al accumulating and nonaccumulating species, respectively. The highest value was measured for *Danaea simplicifolia* (29,300 mg·kg^{-1}), a member of the Marattiaceae. Aluminum accumulators, however, were not randomly distributed and mainly belonged to the fern families Cyatheaceae, Marattiaceae, Gleicheniaceae, and Dryopteridaceae, and the lycophyte family Lycopodiaceae.

Aluminum accumulation was most common in ferns with a tropical-to-subtropical distribution. Among the 281 specimens from our data set that show a tropical-to-subtropical distribution, Al accumulation showed an overall frequency of 40%. Species with a subtropical-to-temperate distribution showed Al accumulation in 32% of the 52 specimens analyzed, and a similar 30% was found for cosmopolitan ferns (n = 20 specimens). The difference in Al concentrations between ferns from these three climate types, however, was not significant (Kruskal-Wallis test; H(2) = 2.39, P = 0.30; Fig. 3A).

Ancestral state reconstruction revealed an overall pattern of uniformity in Al accumulation in ferns, with backbone branches displaying median values throughout the tree (Fig. 2). No large clades exhibited departures from the median values, however, low values tended to occur within the Polypodiales, and high values tended to occur in clades diverging prior to the Polypodiales. Exceptions to both patterns were detected, such as the fairly low values in the eusporangiate fern *Psilotum nudum*, and fairly high values in *Diploblechnum diversifolium*, which belongs to the Blechnaceae in the Eupolypods II clade (Pteridophyte Phylogeny Group I (2016)).

Pagel's λ was significantly different from the model where λ is constrained to 0 (P = 1.74e-12). However the strength of the signal was moderate, with λ = 0.69. The resulting values of Blomberg's
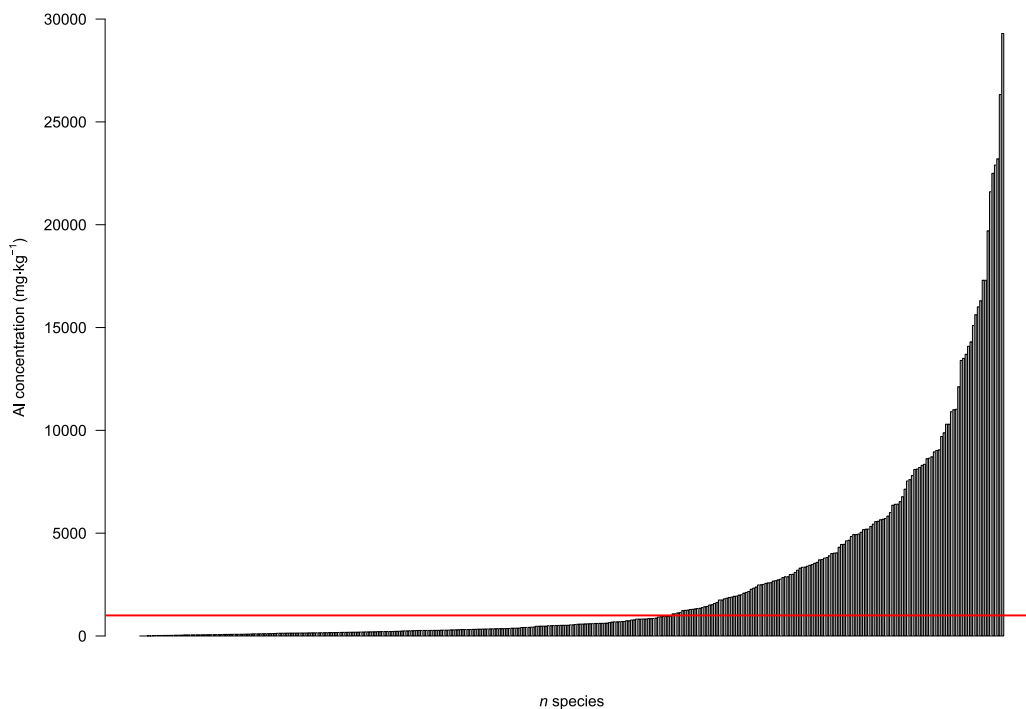

FIGURE 1 Aluminum (Al) concentration in leaves of 313 fern and lycophyte species representing 49 families and 354 specimens. The data were compiled based on novel measurements (n = 171 species, 187 specimens) and literature data (n = 161 species, 167 specimens). The red line indicates 1000 mg·kg^{-1}, which is the general threshold above which plants are referred to as Al-accumulating species.
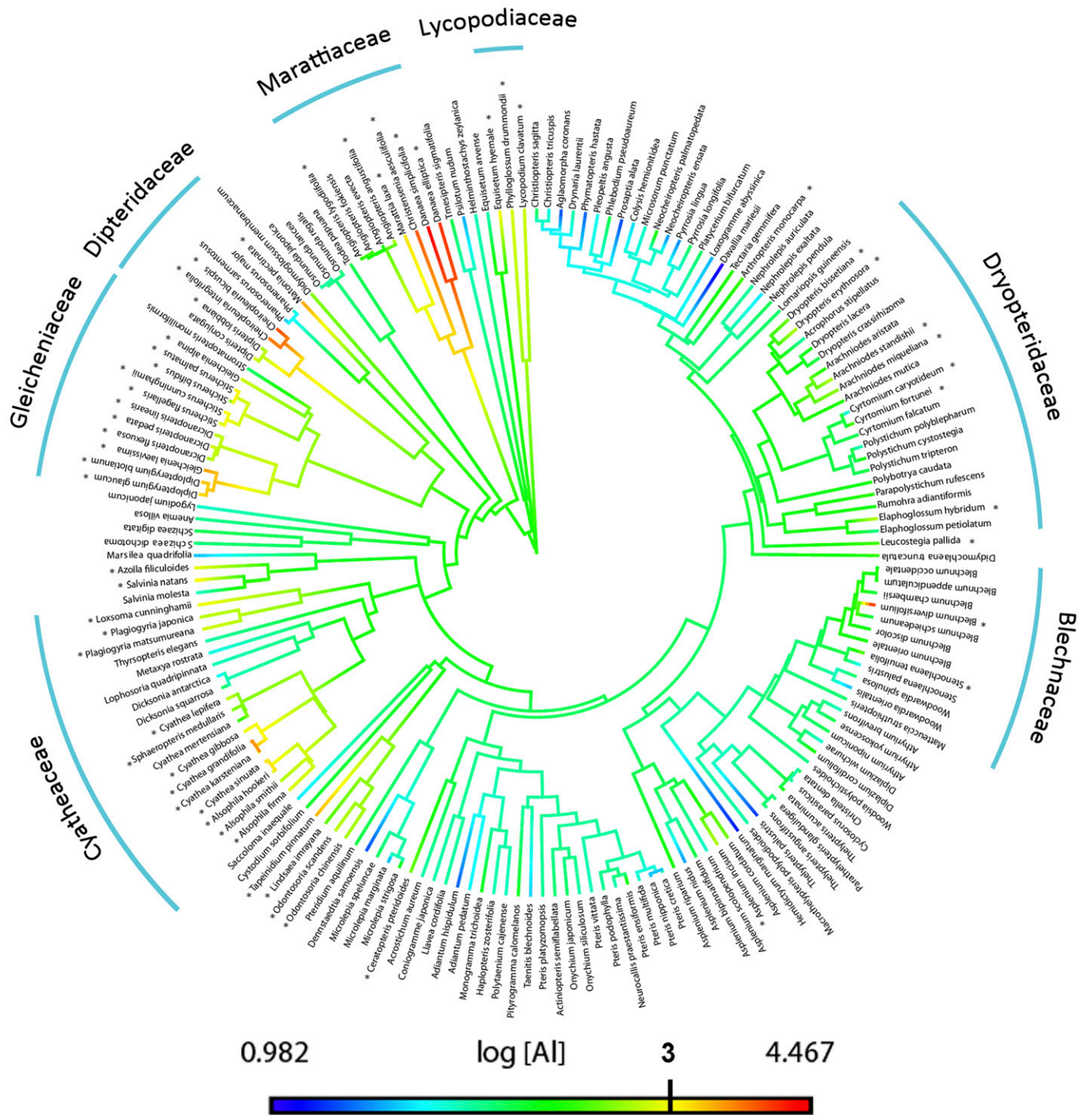
FIGURE 2 Phylogenetic chronogram of 175 fern species for which both sequence data and exact aluminum (Al) concentration data were available. Colors indicate log-transformed values for Al accumulation with blue indicating lower values, and red indicating higher values. The lowest and highest log-transformed Al values are shown above the color scale. Because the threshold of Al accumulation is 1000 mg·kg^{-1} dry weight, the threshold line on the figure is at 3.0. The Al-accumulating species are marked with an asterisk (*).

K (0.08) would indicate high lability of Al, which was consistent with our observations, but the result was not significant ($P = 0.32$).

Epiphytic species had a much lower mean Al concentration (350 ± 429 mg·kg^{-1}, $n = 36$) than terrestrial species (2822 ± 4735 mg·kg^{-1}, $n = 354$, Wilcoxon rank-sum test, W = 7848, $P < 0.01$; Fig. 3B). Our phylogenetic correlative tests indicated that corPagel (AIC = 3389.393) was a better fit than corBrownian (AIC = 3610.71), but neither produced a significant difference ($P = 0.49$ and 0.62, respectively) in Al concentration between epiphytic and terrestrial species.

Al and other elements—We found significant, positive correlations between Al and Fe (Spearman correlation $r = 0.39$, $P < 0.01$), and Al

TABLE 2. Overview of intraspecific variation in aluminum (Al) concentrations in 35 fern species for which more than one specimen was included in the data set compiled, with reference to the source (superscript numbers): 1 = Watanabe et al. (2007); 2 = Chenery 1949b; 3 = Olivares et al. (2009); 4 = Osaki et al. (2003). Al concentrations without reference represent novel data from this study. Species with Al levels recorded above and below 1000 mg·kg⁻¹ (i.e., Al accumulation and nonaccumulation) are shown in bold.

Species	Al (mg·kg⁻¹)
Actiniopteris semiflabellata	258; 273
Adiantum hispidulum	30; 92
Alsophila spinulosa	64; 703; 2880[1]
Angiopteris evecta	416; 580[2]; 5830[2]; 7600[2]
Asplenium cordatum	409; 2592
Asplenium scolopendrium	107; 162
Blechnopsis orientale	243[1]; 942
Blechnum occidentale	270[2]; 481[3]
Blechnum serrulatum	54[4]; 145[2]
Cheiropleura integrifolia	11025; 12118; 16300[2]
Christella dentata	141; 619[3]
Christensenia aesculifolia	14088; 16000[2]
Cyathea gibbosa	3815[3]; 4930[2]
Cyclosorus parasiticus	40; 217[1]
Cyrtomium falcatum	216[1]; 475
Dicranopteris pedata	2763[1]; 3440[1]; 4030
Diplopterygium glaucum	5437[1]; 8620[2]
Dipteris conjugata	195; 3720[1]; 4006[2]; 6412
Equisetum hyemale	141; 8196[1]
Lycopodium clavatum	2725[3]; 3350[1]
Matteucia struthiopteris	193; 251[1]; 361
Metaxa rostrata	109; 283; 340[2]
Nephrolepis cordifolia	202[1]; 1632[3]
Odontosoria chinensis	202; 513[1]; 2272[1]
Pityrogramma calomelanos	313; 608
Plagiogyria pycnophylla	1349; 7800[2]
Platyzoma platyzomopsis	110; 310[2]
Polystichum tripteron	180; 552[1]
Pteridium aquilinum	156[1]; 353; 380; 595
Pteris multifida	78; 188[1]
Ptisana fraxinea	3340[2]; 5195; 5044
Sphaeropteris glauca	1930; 9730[2]
Syngramma alismifolia	65; 325
Thyrsopteris elegans	160[2]; 183
Woodsia polystichoides	48; 420

and Na ($r = 0.17$, $P = 0.02$). Negative correlations were found between Al and P ($r = -0.25$, $P = 0.03$), and Al and K ($r = -0.26$, $P < 0.01$) (Appendix S1; see the Supplementary Data with this article). Magnesium ($r = -0.06$, $P = 0.30$) and Ca ($r = -0.07$, $P = 0.28$) did not show a significant correlation with Al. However, within the nonaccumulators, Al showed only a significantly positive correlation with Fe ($r = 0.38$, $P < 0.01$), but not with any other element (Appendix S2). When analyzing the Al accumulators separately, Al was negatively correlated with Ca ($r = -0.33$, $P = 0.01$) and Mg ($r = -0.39$, $P < 0.01$), but not with other elements (Appendix S3).

Accumulations of As, Cd, Cu, Mn, Ni, and Zn were absent in all specimens analyzed. One exception was *Sceptridium robustrum* (Ophioglossaceae), which was found to be a Ni accumulator with 1719 mg·kg⁻¹. Average values (±SD) for these metals were as follows: As (3 mg·kg⁻¹ ± 13, $n = 164$ specimens), Cd (0.1 mg·kg⁻¹ ± 0.3, $n = 164$ specimens), Cu (17 mg·kg⁻¹ ± 19, $n = 176$ specimens), Mn (275 mg·kg⁻¹ ± 659, $n = 176$ specimens), Ni (42 mg·kg⁻¹ ± 141, $n = 176$ specimens), and Zn (94 mg·kg⁻¹ ± 325, $n = 176$ specimens).

Validation of the aluminon test—Exact Al values were compared for each of the four categories identified based on the aluminon test. The mean Al concentration was 339 ± 271 mg·kg⁻¹ for species with a negative aluminon test (category 0, $n = 73$), 536 ± 591 mg·kg⁻¹ for species belonging to category 1 ($n = 19$), 2727 ± 2453 mg·kg⁻¹ for category 2 ($n = 6$), and 7555 ± 6457 mg·kg⁻¹ for category 3 ($n = 15$), respectively (Fig. 4). No significant difference was found between category 0 and 1 ($P = 1.00$) and between 2 and 3 ($P = 0.32$) (Fig. 4). However, the categories 0 and 1 showed significant differences ($P < 0.001$) to the categories 2 and 3.

DISCUSSION

Evolution of Al accumulation—Our data support the hypothesis that Al accumulation is much more common in ferns than in angiosperms. Its presence in 38% of the species studied in our data set is in line with the 43% estimated by Chenery (1949b), and is considerably higher than the 5% Al accumulating eudicot species. The finding of large numbers of Al accumulating ferns is in clear contrast with the general lack of accumulation of other metals (including Cd, Cu, Mn, Ni, and Zn). An exception of remarkable accumulating properties is *Pteris vittata*, which can take up large amounts of As into the plant tissues (Kertulis-Tartar et al., 2006), a trait which is considered uncommon among fern species.

Moreover, our data confirm that Al accumulation has developed independently in lineages such as Marattiaceae, Dipteridaceae, Gleicheniaceae, and Cyatheaceae, while it is rare in the Polypodiales. These findings confirm earlier work by Chenery (1949b) and Moomaw et al. (1959). Interestingly, Gleicheniaceae include mainly pioneer species, while both pioneers and nonpioneers occur in Cyatheaceae. Pioneer species could be exposed to high levels of Al in acidic soils, which may explain Al accumulation in pioneer species of these families. Exposure to high levels of Al under conditions of low soil pH is especially common in soils with high rates of weathering, soil erosion, land degradation, volcanic eruptions, and landslides (Blume et al., 2010; Brunner and Sperisen, 2013). Interestingly, human modification of the Amazonian landscape was found to create a higher nutrient concentration and more neutral soil pH, which is associated with a decrease in fern species richness (Quintero-Vallejo et al., 2015). There are, however, exceptions because Al accumulation was found to characterize *Danaea* (Marattiaceae), which are not pioneers. Although we found few examples of high Al concentrations among Polypodiales overall, we did find unusually high Al concentrations within the Blechnaceae, namely *Blechnum diversifolium* (= *Diploblechnum fraseri*). Similarly, high concentrations were reported previously in *Diploblechnum fraseri* and *D. lenormandi* (Chenery, 1949b). *Diploblechnum* is not a pioneering species, and thus, these results suggest that there may in fact be multiple and unrelated drivers of Al accumulation in the ferns. It is also possible that Al accumulation has been lost numerous times in the Polypodiales. More data would be required on a wide range of species to determine the evolutionary origins and losses of Al accumulation in a more precise way. Additional information about physiological mechanisms associated with Al accumulation such as translocation factors (i.e., the concentration of Al in aboveground/subterranean organs), and Al detoxification mechanisms in aboveground plant tissues would also be useful to better understand the evolutionary trends of Al accumulation within ferns and lycophytes
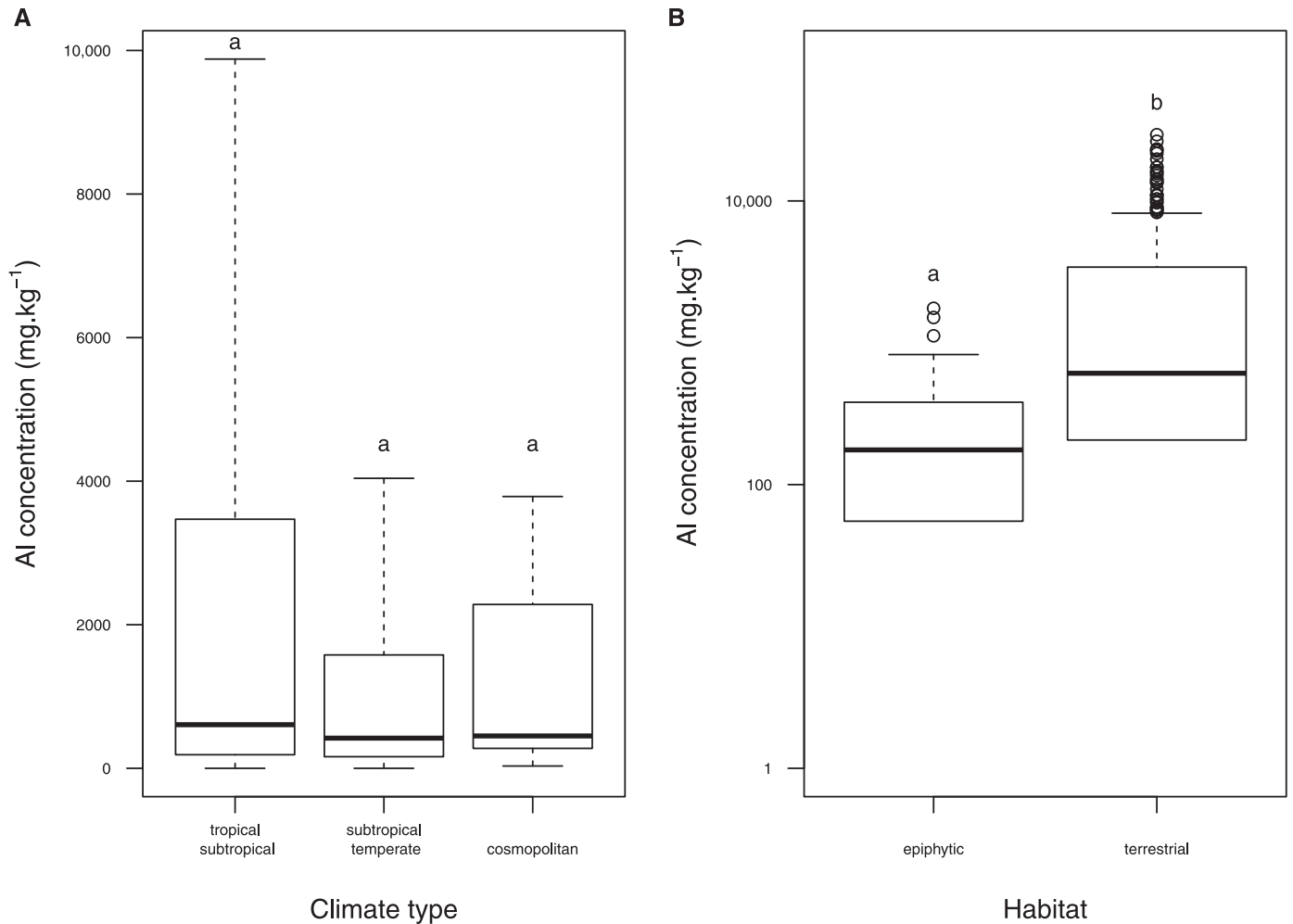
A

B

FIGURE 3 Boxplot showing the aluminum (Al) concentration in milligrams per kilogram (mg·kg^{-1}), with reference to species from three climate types and two growth forms. (A) Species were assigned into three broad climatic zones: (1) tropical to subtropical ($n = 281$ specimens), (2) subtropical to temperate ($n = 52$ specimens), and (3) cosmopolitan ($n = 20$ specimens). (B) Difference in Al accumulation between the terrestrial ($n = 320$ specimens) and epiphytic ($n = 33$ specimens) growth form. The box plots show the median, 25th, and 75th percentiles. Different letters indicate a significant difference between both habitats (Wilcoxon rank-sum Test, W = 7848, $P < 0.01$).

(Jansen et al., 2002, 2004; Watanabe et al., 2007; Olivares et al., 2010; Maejima et al., 2014).

Because the data set analyzed represents a compilation of data from various laboratories, minor quantitative differences could be caused by the methods applied to quantify Al concentration in leaf tissue (i.e., ICP-MS, GF (graphite furnace)-AAS, MP-AES (Microwave Plasma-Atomic Emission Spectrometry), photometry). It is unlikely, however, that taxa will be classified as accumulators based on a particular method and as nonaccumulator based on another method. With the exception of seven species that showed intraspecific Al concentrations both below and above the threshold of 1000 mg·kg^{-1}, all other 28 species for which we had more than one measurement were consistently accumulating or nonaccumulating species, regardless of the origin of the data (Table 2).

The most likely explanation for intraspecific variation in Al concentration is that potential differences are caused by sampling location, soil pH, or edaphic characteristics, which are known to affect Al accumulation in some angiosperms, especially facultative Al accumulators (e.g., Schmitt et al., 2016a, b). If high levels of Al ions

are available to a plant because of low soil pH, the Al concentration in aboveground plant organs can be higher than when a plant is growing in a soil with a higher soil pH and lower amount of Al ions (Haridasan, 1988; Watanabe et al., 2005a; Schmitt et al., 2016a, b). The potential effect of soil acidity and availability of soluble Al forms is also suggested by the finding that higher relative frequencies of Al accumulation occur in tropical and subtropical rather than in temperate climate zones. Tropical soils are frequently acidic and are also known to show a higher frequency of Al accumulating angiosperms than soils in temperate environments (Jansen et al., 2002; Brunner and Sperisen, 2013). While Al accumulators take up high levels of Al by definition, irrespective of the soil pH and amount of exchangeable Al, Al accumulators may well show Al levels below the threshold of 1000 mg·kg^{-1}. Therefore, considering that we do not have soil data available, the Al accumulating species reported in our data set represent true, proven accumulators. The absence of Al accumulation in a particular species, however, does not indicate that this species may not be able to show Al accumulation when growing under certain soil conditions. As far as we know,
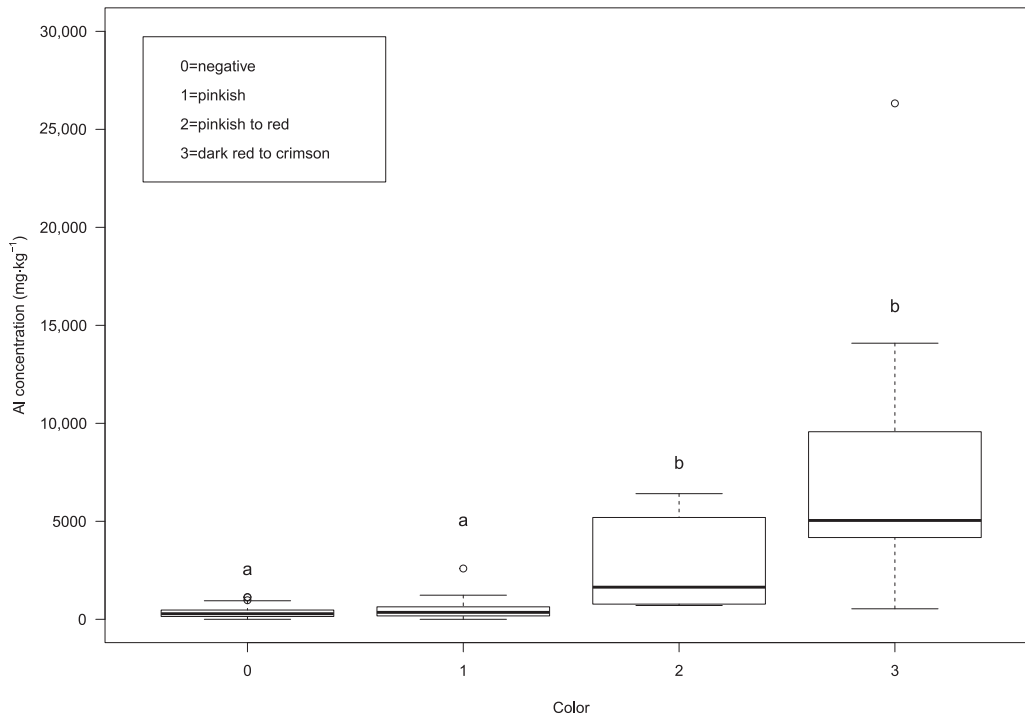
FIGURE 4 Comparison of the semiquantitative test based on Chenery (1949b) with exact concentrations of aluminum (Al) for fern leaves of 105 species. Four arbitrary categories were distinguished based on the color reaction with Chenery's aluminon test (color 0, 1, 2, 3) as the reaction of the test solution intensifies with higher Al concentration in the tissue tested. Samples were tested on the same leaf material from a single specimen for 105 species. The box plots show the median, 25th, and 75th percentiles and individual points show outliers. Different letters indicate significant differences (pairwise Wilcoxon rank-sum test with Bonferroni correction, $P < 0.05$).

growth experiments to determine Al uptake under variable concentrations of exchangeable Al and soil pH have not been applied to ferns. Therefore, we are unable to identify facultative Al accumulators in our data set.

Terrestrial vs. epiphytic ferns—Our results show that epiphytic fern species show significantly lower Al concentrations, whereas terrestrial ferns show an overall higher concentration in their tissues (Fig. 3B). However, this difference was not significant if a phylogenetic correction was applied to the data set. Interestingly, some epiphytic species such as *Didymoglossum membranaceum* and *Elaphoglossum sporadolepis* had Al concentrations >1000 mg·kg⁻¹ and could thus be classified as Al accumulators. The specimens of these species might have been growing on epiphytic soils, which can form on thick, wide tree branches, and which can have a similar nutrient composition but lower pH than terrestrial soils (Watkins and Cardelús, 2012). Therefore, a low pH could explain why epiphytes can take up high concentrations of Al, but field observations and detailed measurements of terrestrial and epiphytic soil are required to test this idea.

Aluminum and other elements—The role of Ca in understanding Al toxicity or amelioration has been investigated in various angiosperm species (Rengel, 1992; Ryan and Kochian, 1993; Ryan et al., 1994). High Al ion concentrations may reduce the uptake of Ca in the root tissue and was suggested to be one explanation for Al toxicity in plants with a low Al tolerance (Rengel, 1992; Ryan and

Kochian, 1993; Ryan et al., 1994; Reid et al., 1995). However, there are also positive correlations between Al and Ca levels in angiosperms, including Al accumulators (Masunaga et al., 1998; Schmitt et al., 2016a). A potential explanation for this positive correlation in Al accumulators could be the high concentration of oxalate in leaves, which may regulate not only Ca by producing Ca oxalate, but also detoxify Al by forming Al-oxalate complexes (Ma et al., 1997, 1998; Morita et al., 2008; Watanabe et al., 1998, 2005b; Maejima et al., 2014). The negative correlation between Ca and Al as observed in this paper (Appendix S3A) is not in line with this detoxification idea and may suggest that detoxification mechanisms differ between angiosperms and ferns.

Besides Ca, Al may have an effect on the uptake of Fe, which is also an essential plant nutrient. Aluminum has been found to reduce the Fe toxicity in plants (Watanabe et al., 2006; Hajiboland et al., 2013). It has also been shown that Al can reduce the uptake of Fe, which may lead to Fe deficiency (Foy, 1988). The positive correlation between Fe and Al in our data set (Appendix S1C, Appendix S2C) suggests neither an inhibition of Fe uptake, nor a reduced Fe uptake with elevated Al concentrations. More research is needed to understand the potential interactions between Al and Fe in ferns by conducting cultivation experiments with different levels of Al and Fe. Soil properties in combination with growth experiments in hydroponic solutions would also be required to provide additional insight in potential correlations between Al and other nutrients (including Na, K, P, and Mg) within ferns.

Validation of the aluminon test—The differences between the arbitrary categories 0 and 1, and categories 2 and 3 indicate that a concentration of 1000 mg·kg⁻¹ Al in plant tissue can be determined using the aluminon test. For an estimation of the Al concentration in dried (or fresh) plant material, this semiquantitative test can be used to quickly determine Al accumulators and nonaccumulators using a fast and easy method. This result is in line with an earlier, preliminary comparison between the aluminon test and exact Al concentrations for 10 Rubiaceae species (Jansen et al., 2003). Furthermore, the test solution is also used as a dye for studying the localization of high Al levels in plant tissues (Haridasan et al., 1986; Haridasan, 2008; Campos et al., 2014). However, Clark and Krueger (1985) critically investigated the aluminon solution with regards to other elements and reported that staining patterns similar to Al can be found for other elements, especially Fe.

CONCLUSIONS

Our findings indicate that Al accumulation is much more common in ferns than in angiosperms. In particular, our phylogenetic analyses show that this trait is prominent in numerous fern lineages, especially Marattiales, Gleicheniales, and Cyatheales. Based on the overall framework presented, future research should focus on the physiological mechanisms associated with internal detoxification and exclusion mechanisms. More work is also needed to explore to what extent Al accumulation within ferns and lycophytes is affected by environmental conditions, especially soil properties, and whether Al behavior strategies are associated with ecological distribution patterns.

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