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The Velamen Radicum Is Common in the Genus *Anthurium*, Both in the Epiphytic and Terrestrial Species

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Abstract: The velamen radicum, a rhizodermis that consists of dead cells at maturity, is often described as typical for epiphytic aroids. Such claims are surprising on two grounds: (1) there are hardly any data on this trait for aroids and (2) the link between a velamen and epiphytic growth has recently been challenged in general. We performed an anatomical and histological study with 82 *Anthurium* species and analyzed the occurrence of a velamen in regard to habit (epiphytic vs. terrestrial) and phylogenetic relatedness. Almost 90% of both epiphytic and terrestrial species had a velamen. The number of cell layers comprising this tissue were also very similar in both groups. The most likely interpretation of the phylogenetic tree suggests that a velamen is not ancestral in *Anthurium*. It was gained once and has been lost several times during diversification of the genus. Our results are an important contribution to the current discussion on the possible function of the velamen. While there is some experimental evidence for its importance for epiphytic plants, its role in terrestrial plants is completely unresolved.

Keywords: *Anthurium*; functional morphology; growth habit; roots; velamen radicum

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1. Introduction

The velamen radicum is a spongy uni- to multiseriate rhizodermis that consists of dead cells at maturity and is bordered internally by a hypodermis, which is termed an exodermis [1]. Almost two centuries ago, Link [2] originally described the velamen in the roots of epiphytic orchids as a macroscopically visible whitish parenchyma that ensheaths the root. Link's German term for this tissue ("Wurzelhülle") was later latinized by Schleiden [3] to velamen radicum.

The velamen is most frequently mentioned in the context of epiphytic growth, especially in orchids and aroids [4,5]. The standard explanation for the frequent observation of the velamen among plants in the epiphytic habitat is its absorptive function in terms of water and nutrients [6], but the velamen may also be associated with other functions like UV protection [7]. However, the claim of a tight association of the velamen with epiphytism ignores that this tissue has also been reported many times in terrestrial life forms like geophytes and hemicryptophytes [8–10]. Actually, the velamen seems to be quite commonly found among terrestrial monocotyledons as has been demonstrated in a recent revision of the literature [11]. These authors found at least one report of a species with a velamen in more than a quarter of the 76 families of monocots, and 13 of these 23 families are entirely terrestrial, which precludes the possible explanation of velamentous terrestrial species being derived from epiphytic ancestors.

This paper questions another, more specific claim that has been made in numerous textbooks and monographs; the claim that the velamen is associated with epiphytic orchids and aroids (e.g., [4,12–14]). While the distribution of a velamen within Orchidaceae is

well documented [15], the actual basis for this claim in regard to Araceae is quite thin. In their review, Zotz, Schickenberg and Albach [11] were only able to find evidence for the occurrence of a velamen in 13 *Anthurium* species and very few species of other genera in the family, with a majority of the observations being more than a century old [16]! Considering that *Anthurium* is a hyperdiverse genus with more than 1000 species [17], the current evidence is thus certainly not sufficient to make any such claims. Not surprisingly then, neither Cusimano et al. [18], in their comparison of morphological patterns and molecular phylogenies within the family Araceae as a whole, nor Carlsen and Croat [19], in their phylogenetic analysis of the genus *Anthurium*, included the velamen as a trait.

In the current study, we investigate the occurrence of a velamen in *Anthurium* species in more detail and in relation to their growth habit, i.e., epiphytic vs. terrestrial growth. We studied the occurrence of velamen for a large proportion of the species included in the phylogeny of Carlsen and Croat [19], supplemented by a number of additional species, and conducted a parallel phylogenetic analysis. Based on this phylogeny, we investigated the suggested link between the velamen and epiphytism in *Anthurium* by including information on growth habits from the most recent tally of epiphytes among vascular plants [20]. We also studied differences in velamen thickness since two studies of epiphytic orchids reported an increase in the number of cell layers in drier habitats [21,22]. With our analysis, we can show that a velamen is indeed common in the genus, but that both its occurrence and its structure are largely independent of epiphytic or terrestrial growth.

2. Materials and Methods

Most of the material for this study was obtained from the collections of the Missouri Botanical Gardens, but we also included root material from a number of other sources (Table S1). Upon arrival in Oldenburg, roots were rinsed and cleaned carefully in tap water. Transverse sections were cut by hand at a distance of approx. 3 cm from the root tip and prepared for histological and histochemical analysis.

Growth habit: Species were either labeled "terrestrial herbs" (including climbing species rooted in the soil such as *Anthurium clidemiodes* Standl.) or "epiphyte" using EpiList 1.0 [20], which also included hemiepiphytes such as *A. clavigerum* Poepp. Endl., which are characterized by an epiphytic stage in their early ontogeny [23]. Treating growth on trees or soil as a binary trait has obvious shortcomings because a considerable number of species may use both substrates, i.e., are facultative epiphytes sensu Ibisch [24]. Examples are *A. cubense* Engl., *A. maculosum* Sodiro, *A. mindense* Sodiro or *A. schlechtendalii* Kunth. Although this issue could possibly be resolved, as was recently shown for the family Hymenophyllaceae [25], the necessary literature survey is very time consuming and was beyond the scope of this study. An additional problem arises since terminology, particularly of creeping species, is often used very inconsistently [26]. This is most relevant in the case of *A. clidemioides* and *A. flexile* Schott, which represent the earliest-diverging lineage within the genus [19]. These species have been described either as epiphytes [27,28], hemiepiphytes [29], adpressed climbers [27], or vines [30,31]. Here, we treated both species as vines, i.e., terrestrials.

Histochemistry: To distinguish a living rhizodermis from a dead velamen the vital test with Triphenyl Tetrazolium Chloride (TTC) was used, with which the viability of cells and tissues can be tested [32,33]. In this test, TTC serves as a redox indicator to identify living, metabolically active cells by indicating cellular respiration of mitochondria. When applied on a living tissue, the colorless indicator is reduced by active dehydrogenases to the red 1,3,5-triphenylformazan. Freshly cut transverse sections from living roots were incubated in Eppendorf cups (1.5 mL) in 1% TTC at 32 °C at least for 8 h in the darkness and stored at 4–6 °C overnight. Transverse sections then were transferred on microslides in H_2O under cover glasses for microscopy. A mature velamen will not stain.

Histology: For histological examinations approx. 0.5 cm root parts were infiltrated and embedded in TECHNOVIT® 7100 (Kulzer GmbH, Wehrheim, Germany). TECHNOVIT® is a polymerization system based on 2-hydroxyethyl methacrylate and polymerizes transparently. The embedding protocol provided by the company was followed, i.e., samples were fixed in AFE (stock: 5 mL Acetic Acid, 5 mL 37% formaldehyde, 90 mL 70% ethanol), dehydrated in an ascending alcohol series (70%, 70%, 90%, 100% EtOH, changed every two hours, last step over night), pre-infiltrated in 50% EtOH/50% basic solution TECHNOVIT® 7100 at 4 °C overnight and afterwards infiltrated for at least 48 h to up to one week at 4 °C. For polymerization the infiltrated samples were arranged carefully in Teflon Histoform embedding cavities (Size: S, Kulzer GmbH, Wehrheim, Germany), filled with 0.7 mL TECHNOVIT® polymerisation solution each, and within one to seven days blocked with TECHNOVIT 3040 in Histoblocs (Kulzer GmbH, Wehrheim, Germany). Thin sections (5–8 µm) of the hardened blocks were cut by HISTOBLADES (Kulzer GmbH, Wehrheim, Germany) with a microtome (Reichert-Jung SUPERCUT 2050, Wetzlar, Germany) and collected in a water bath at 20–25 °C. Thin sections were placed on microscope slides (VWR®) and air-dried for at least two hours. The samples were stained in 0.05% toluidine blue aq. (toluidine chloride 1272, Merck KGaA, Darmstadt, Germany) for 5 min, washed in three water baths for 20 sec each and air-dried overnight. Microslides were mounted in ENTELLAN® (107961, Merck KGaA, Darmstadt, Germany). The samples were examined under a light microscope POLYVAR Type 300602 (Reichert-Jung, Wetzlar, Germany). Imaging was performed using the connected Camera OLYMPUS UC30 (Olympus, Hamburg, Germany). In combination with the histochemical evidence, we counted the number of cell layers that make up the velamen of four samples in a randomly chosen part of each sample. Variation was generally small and only means are reported.

DNA sequencing: In total, our dataset consisted of 134 accessions compiling 123 *Anthurium* taxa, representing approximately 13% of the published 950 *Anthurium* species [34]. Moreover, our sampling strategy ensured that all currently recognized 19 sections of *Anthurium* were represented, making our dataset comprehensive and suitable for exploring phylogenetic relationships within the genus. A total of 101 specimens were sourced from a previous study by Carlsen and Croat [19]. To augment our dataset and enhance the representation of *Anthurium* diversity, we included 33 additional *Anthurium* specimens (Table S1).

Genomic DNA was isolated from about 20 mg of silica-dried leaf tissue by using the innuPREP Plant DNA Kit (Analytic Jena AG, Jena, Germany), following the manufacturer's instructions. The DNA concentration was measured with a TECAN infinite Pro 200-F and NanoQuant Plate™ (Männedorf, Switzerland), and each DNA sample was diluted to 10 ng/µL. Three plastid regions and one nuclear region were sequenced for this study. These plastid regions are *trn*G intron [35], the *trn*H-*psb*A [36] and *trn*C-*ycf*6 [35] intergenic spacers. The nuclear region is the *chalcone synthase* (*CHS*) gene [19]. The DNA amplification reactions for the polymerase chain reaction (PCR) contained 2 µL of diluted DNA template, 2.5 µL of 10× reaction buffer, 0.5 µL of 10 mM dNTPs mix, 1 µL of 10 pmol/µL stock for each primer, 0.2 µL of Taq polymerase (5 units/µL) (NEB Biolabs, Ipswich, MA, USA) and water to a final volume of 25 µL. Thermocycling conditions included a 2 min denaturation step at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and a final 8 min extension at 72 °C. The same reaction mixes and cycling profiles were used for the three chloroplast (cpDNA) regions. The PCR protocol for the *CHS* region contained 3 µL of diluted DNA template, 5 µL of 10× reaction buffer, 1 µL of 10 mM dNTPs mix, 2 µL of 10 pmol/µL stock for each primer, 0.8 µL of Taq polymerase (5 units/µL) (NEB Biolabs, MA, USA), 5 µL of betain as an enhancer, and water to a final volume of 50 µL. Thermocycling conditions included a 2 min denaturation step at 94 °C, followed by 40 cycles of 60 s at 94 °C, 60 s at 52–60 °C, 2 min at 72 °C, and a final 7 min extension at 72 °C. The PCR products were checked using a 2% agarose gel electrophoresis. The PCR products sequenced by the external sequencing service of LGC Genomics (Berlin, Germany).

DNA sequence analysis: Sequence editing and assembly was performed in Geneious 9.1.8 (http://www.geneious.com/, (accessed on 1 December 2023)). A _"de novo"_assembly was conducted by Geneious-Assembler. Assembled sequences were edited manually. The sequences were aligned using MUSCLE as incorporated in Geneious [37]. The alignment was checked visually and corrected where the algorithm failed to identify gaps. The aligned DNA regions were concatenated in Geneious. The sequences of the four DNA regions were analyzed separately and concatenated using maximum likelihood in RAxML-NG v.1.0.3 [38] with the GTR with empirical base frequency parameters and 100 bootstrap replicates.

Stastistical and comparative analyses: Statistical tests were performed with the software R 4.02 [39]. Differences in the proportions of terrestrial and epiphytic species with a velamen were assessed with a Chi^2-test; differences in the number of cell layers with a two-sided *t*-test. Character evolution was inferred using the R-package 'ape' [40] and visualized in R using the package 'phytools' [41]. The phylogenetic signal for the discrete characters velamen presence/absence and epiphyte/terrestrial was quantified using the δ-value [42]. Significance was determined based on 100 iterations. The phylogenetic signal of the continuous character number of velamen layers was measured using Blomberg's K [43] in the R package 'picante' [44] using the command phylosignal.

3. Results

Our sample of 82 species for the anatomical and histological analyses covers the entire genus with all the clades identified by Carlsen and Croat [19]. The proportion of epiphytes (about 70%) in the sample is comparable to their relative importance in the genus as a whole [20]. A velamen was found in 72 of the species, i.e., in close to 90% of all taxa. The proportion of species with a velamen was slightly higher in epiphytes than in terrestrials (Chi^2 [df = 1, N = 82] = 4.6, $p = 0.03$), but differences in the number of cell layers that composed the velamen were unrelated to growth habit. Meanwhile, the number of cell layers varied by almost an order of magnitude from 1 (e.g., *A. antioquiense* Engl.) to 9 cell layers (*A. vittariifolium* Engl.), and the average number of cell layers of the epiphytic and terrestrial species was very similar (3.3 ± 1.9 vs. 2.6 ± 1.9 cell layers (means ± SD) in the terrestrial and epiphytic taxa, *t*-test, $p = 0.15$).

Statistical analyses ignoring phylogeny may be biased by the non-independence of species and the unequal evolutionary time between various species of the dataset. Therefore, we constructed a phylogenetic hypothesis based on four DNA regions to understand the evolution of growth habit and velamen in the genus. The final alignment of four DNA regions and 142 taxa included 4657 positions (CHS: 2242, *psb*A-*trn*H: 586, *trn*G intron: 744, *trn*C-*ycf*6: 1085) with 72% invariant sites (69%/50%/9%/43%) and 52% gap positions (70%/23%/67%/65%). Separate analyses differed from the combined analysis especially in the lack of resolution and lower support of branches (results not shown). However, for four species (*A. andreanum* Lind., *A. pentaphyllum* G.Don, *A. salvinii* Hemsl., *A. cuspidatum* Matuda) the positions differed markedly between plastid DNA and CHS and the two regions were therefore analyzed separately in the final combined analysis as if they were from two separate individuals. A similar strategy was used for *A. thompsoniae* I.Arias, which had different positions using different plastid regions. The tree (Figure S1) resembles the one previously published by Carlsen and Croat [19] and contains the same well-supported clades with the 16 clades recognized there were retrieved again. Our 47 new accessions group with these clades or other species from previous analyses but do not form new, well-supported clades. Most species fall into the clades anticipated by previous sectional classification based on morphology. *Anthurium gymnopus* Griseb. from the previously unsampled *A.* sect. *Gymnopodium* appears close to *A. venosum* Griseb. The recently described *A. thompsoniae* falls into clade 8 (sect. *Pachyneurium* p.p.). Other previously unplaced taxa are *A. ernestii* Engl. (clade 12, sect. *Pachyneurium* p.p.), and *A. pentaphyllum* (clade 3, sect. *Dactyllophyllum*) [45]. The latter is here inferred to be a hybrid based on the different positions of CHS (in clade 3) and cpDNA sequences (in clade 4).

Two more samples with strong incongruence between plastid DNA and CHS are *A. salvinii*, which is split between clade 8 (CHS) and clade 13 (plastid DNA), and *A. cuspidatum*, which does not form a clade with the other sample of *A. cuspidatum* in clade 15 but with cpDNA being distantly related to that clade and CHS showing a relationship with clade 12. A group of species formerly associated with *A.* sect. *Pachyneurium* are together found in a position distant from other members of the section close to clade 8 (*A. wagenerianum* K.Koch & C.D.Bouché, *A. cubense* Engl., *A. schlechtendalii* Kunth). Two species, *A. rubrinervium* G.Don and *A. oxycarpum* Poepp., were in unexpected positions, which will be discussed below.

Based on these analyses of character evolution, a velamen has been gained once in the genus and has subsequently been lost nine times, independently in almost each species without velamen (Figures 1 and S2). Similarly, species with an epiphytic and terrestrial habit are scattered over the phylogenetic tree with considerable uncertainty in the reconstruction of the ancestral habit (Figures 1 and S3). The number of velamen layers was ancestrally low with 1–3 but in several clades a higher number of cell layers has been gained in parallel (Figure 2). None of the three analyzed characteristics (habit, presence of a velamen, number of velamen cell layers) demonstrated significant phylogenetic signal. In particular, growth habit is highly labile ($\delta = 0.44$, $p = 0.71$) with presence of velamen showing stronger dependence on phylogeny ($\delta = 10.41$, $p = 0.13$) as does the number of velamen layers ($K = 0.14$, $p = 0.17$).

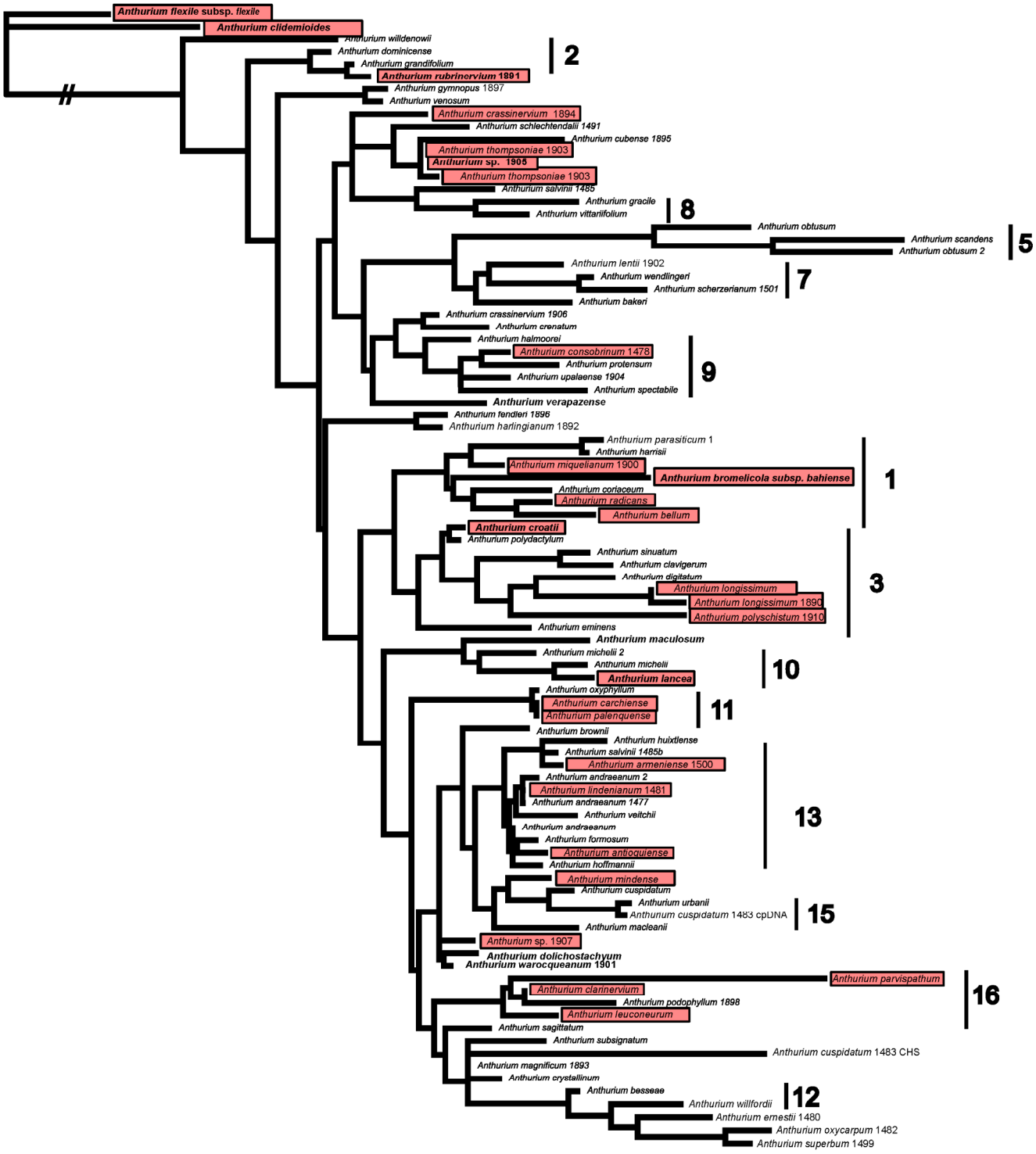

Figure 1. Phylogenetic tree of *Anthurium* based on the concatenated dataset with all taxa pruned from the tree for which no information on the velamen is available. Taxa with species names in bold possess no velamen. Taxa in red boxes are terrestrial. The numbers behind the species names indicate sequences newly generated in this study (Table S1). The bold numbers indicate clades as identified by Carlsen and Croat [19].

Figure 2. Phylogenetic tree of *Anthurium* based on the concatenated dataset with all taxa pruned from the tree for which no information on the number of cell layers of the velamen is available. The number of cell layers, ranging from 1 to 9, is depicted in colors from red to blue using the contmap command in the R package phytools [41]. The numbers behind the species names indicate sequences newly generated in this study (Table S1).

4. Discussion

Orchids and aroids have long been the textbook examples of families with numerous velamentous species [4,46–48]. However, unlike orchids, for which a detailed investigation of the occurrence within the family has been published decades ago [15], a similar study for aroids, particularly for the genus *Anthurium*, has been missing. The present study fills this gap and clearly shows that a velamen can be found in the vast majority of *Anthurium* species.

The long-standing notion that a velamen radicum is almost exclusively associated with epiphytic growth has been refuted recently by [11] in the course of a thorough revision of its occurrence in the plant kingdom. Our current data set of 82 *Anthurium* species is in line with their findings and further questions the explicit or implicit depiction of this root tissue as an *adaptation* of epiphytes [5,49]. The cited statement can be criticized on two grounds. First, a velamen is clearly not restricted to plants in tree crowns, but is almost as common in terrestrial as in epiphytic members of the genus. Second, even if a velamen is found slightly more commonly among epiphytic plants, this represents no evidence for an adaptation. An adaptation is both "the *process* whereby the members of a population become better suited to some feature of the environment" and the resulting "*characteristic* that has evolved by natural selection" [50]: the mere functionality of a trait does not distinguish an adaption from an exaptation. Such a lax use of the term adaptation is common in other areas of biology as well. For example, although silification in grasses

is often depicted as an "adaptation" to protect plants from herbivory by grazing animals, a recent review states that it is probably an exaptation [51].

Understanding the functionality of the velamen in epiphytic and terrestrial habitats could help to decipher the actual evolutionary scenarios, but—in spite of considerable progress, particularly in the last decade—the role of this tissue in nutrient and water relations, in UV protection, or in thermal protection in the epiphytic habitat is far from fully resolved [6,7,52–54], or basically still an entirely open question in terrestrial habitats [55]. There are two reports that demonstrate a negative correlation of the number of velamen cell layers and in situ water availability [21,22], noting that a multi-layered velamen offers greater protection against desiccation and a larger dead space to extend the time for water and nutrient uptake [56]. Since water supply is typically described as the major challenge for epiphytic growth, we expected—if not differences in the *occurrence* of a velamen—at least differences in velamen structure with fewer layers in terrestrial taxa. This expectation did not bear out.

An analysis of the connection of the velamen radicum with the epiphytic vs. terrestrial habitat would also benefit from a more fine-grained categorization of the growth habit of the included species. For lack of a better alternative, we used Epilist 1.0 [20] to categorize species as epiphytes or terrestrials. Zotz et al. [20] emphasized that such a binary categorization obscures the fact that the tendency to grow on trees varies strongly among species, from the occasional epiphytic individual in the case of many facultative epiphytes to (almost) exclusive epiphytic occurrence. The problem of categorizing species of *Anthurium* according to habit also becomes apparent in the difficulty of reconstructing the ancestral character state and the frequent transitions between the binary categories (Figure S3). There have been recent efforts to capture this variation by conceptualizing growth of plant species as epiphyte, lithophyte or terrestrial in an analogous way as in Grime's CSR-space [25], but quantitative data for such an ordination are not available for *Anthurium*. Moreover, the literature is full of ambiguous information on growth habits, and it is often impossible to distinguish actual biological variation from terminological confusion (e.g., [57]). Dubious growth habit assignments became particularly problematic in the case of *Anthurium flexile* and *A. clidemioides*. Information in the literature on the growth habits of these two species is highly variable (see Materials and Methods). Considering that these species represent sisters to the rest of the genus, this assignment influences strongly whether epiphytic growth is seen as an original condition in the genus or whether both epiphytic and terrestrial growth are derived from an original climbing habit. Based on expert advice (T. Croat, T. Krömer, A. Cascante-Marin, M. Carlsen, pers. comm.) we conclude that the latter is most likely the correct interpretation. The absence of a velamen in both species suggests that it is correlated with the climbing habit and the absence is also ancestral.

Finally, we note that our inference is based on an incomplete sampling of the approximately 1000 species in the genus. Our sampling of species broadly across the genus limits the likelihood that our sampling is biased with respect to growth habit or presence of velamen. We also note that our phylogenetic hypothesis is limited in both the number of species and the loci across the genome. Surprising phylogenetic results, such as *A. oxycarpum*, which was expected to be in clade 8, 10 or 16 by Carlsen and Croat [19] but was found near clade 12, and *A. rubrinervium*, which is considered a synonym of *A. sagittatum* G.Don [45], but is here only distantly related to that species, will require further examination. Further sampling of taxa and loci across the genome will provide us with more robust phylogenetic hypotheses and also allow us to infer better the ecological circumstances for evolutionary switches between terrestrials and epiphytes and changes in the presence of a velamen.

In conclusion, we present the first major survey of the occurrence of the velamen radicum in the genus *Anthurium*. Provided the absence of a velamen is ancestral in the genus as discussed above, the trait was gained once. Although common in the large majority of extant species, it was also lost several times independently in the continued diversification of the genus. Its prevalence in both epiphytic and terrestrial species contradicts the frequent

stated association of the velamen and epiphytic growth and calls for studies investigating the functional relevance of the velamen, particularly in terrestrial plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16010018/s1, Figure S1: Phylogenetic tree of *Anthurium* based on the concatenated dataset with all taxa included. The numbers on the branches provide maximum likelihood bootstrap support. The numbers behind the species names indicate sequences newly generated in this study (Table S1). The bold numbers indicate clades as identified by Carlsen and Croat [19]; Figure S2: Phylogenetic tree of *Anthurium* based on the concatenated dataset with all taxa for which information on velamen is included. Branches for which velamen is inferred to be present are red, those without velamen are black. Figure S3: Phylogenetic tree of *Anthurium* based on the concatenated dataset with all taxa included. Pie charts provide probability for ancestral node to be epiphytic (blue) or terrestrial (red); Table S1: List of voucher specimens and GenBank accession numbers for sequences used in the phylogenetic analysis; Table S2: Character coding of growth habit and occurrence of velamen as used in our analyses. Life form (0 = terrestrial, 1 = epiphytic), Velamen, CL = number of cell layers of the velamen.

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