nature portfolio

Peer Review File

Tracing the evolutionary and genetic footprints of atmospheric tillandsioids transition from land to air

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Please see attached file.

Reviewer #2

(Remarks to the Author)
This manuscript seeks to understand innovations in the evolution of epiphytic plants. By combining multiple 'omics technologies with a highly resolved phylogenetic tree, the authors provide insight into the evolutionary genetics involved in important traits that enabled the transition from soil to air lifestyle. I was asked to comment on the microbiome analyses, and so my review is focused on those sections.

Overall, there is insufficient detail provided by the authors to evaluate the microbiome analyses.

My key conceptually concern is with the claim that the relative abundance of "phyllospheric bacteria" differ between tank and atmospheric types (lines 557-558). It seems like the bacterial community is being partitioned into a phyllospheric and non-phyllospheric community. As leaf tissue was used to generate the 16S data, then all OTUs are from the phyllosphere. So how the microbiome is being separated into these two components needs additional justification and detail. The network analysis (supp. fig. 13 and result lines 561-565) does not say anything about "dominant genera". Finally, the conclusion that nitrogen fixing bacteria are an important aspect of tillandsioids biology that underlie adaptation to the air lifestyle is unsupported. As the authors state, these nitrogen fixing bacteria, like Sphingomonas, are commonly found on diverse plants, and so it's not clear if there is some sort of special enrichment for nitrogen fixing bacteria; this would really need additional work. Only one species of tank type was used in the metagenome analysis, and so connecting these results further to the distinction of tank and atmospheric is difficult. You could probably mine the Pacbio HiFi genomes for more detailed metagenomic information to broaden the comparison.

Another key point of concern is that it's not clear how the growing environment is controlled for in the microbiome samples. I recognize the difficulty of growing this different plant species, but whether the plants were grown at the same time or under the same environmental growth conditions could confound the microbiome analysis. More clarity is needed.

I next organize my comments to provide feedback based on methods and figures.

For 16S rRNA profiling, it is unusual to see data presented as OTUs. Most microbes studies have used ASVs for at least the past 5 years. There is nothing inherently wrong with using OTUs, but this rationale should be explained.

Variation in read depth needs to be clarified. Rarefaction curves are needed to show that communities were sufficiently sampled. Given that V3-V4 region was used, chloroplasts often comprise a substantial amount of reads, without using peptide nucleic acid clamps. Presumably plastid sequences were removed from analysis, but it is not clear what the authors did (and rarefaction should be done after chloroplast removal). I can see that "f__mitochondria" were identified as differentially abundant in the Fig. 7C cladogram, which suggests that this kind of filtering was not applied. Plastids are

normally removed from 16s analysis.

Whether or not the data was rarefied before PCoA or LEfSe analysis is important because variation in read depth across samples needs to be accounted for in the statistical model. Similarly, it matters if the rarefaction curve has reached saturation. This needs to be clarified.

Furthermore, for the OTU differential abundance, it is normal that some pre-processing/filtering is applied to the data set (i.e., include only OTUs that occur across a majority of samples across categories). Again, it's important to know if the data was rarefied or not before differential abundance analysis and how this was handled. Similarly, there is no method described that generated the network analysis in Supp. Fig. 13.

A similar lack of important methodological detail also exists for the metagenomes.

Some specific comments on the microbiome figures:
Fig. 7A: SEM images are not super instructive as which plant is not labelled, and while the arrows point to "microorganisms", which could include both fungi and bacteria, the data are only on bacteria. I did not find this figure to be particularly useful.

Fig. 7B: "others' category makes up most of a lot of samples. what is in the others?

Fig. 7C: I don't really understand what this cladogram is supposed to show. I'm guessing the non-highlighted nodes are not differentially abundant between tank and atmosphere type? It just isn't intuitive about how to relate the cladogram to differential abundance. The heat map in supp. fig. 14 is maybe more intuitive? I also don't understand what "phylogenetic distribution from phylum to genus of the microbiota" means here. I'm confused because the tree was based on OTU sequences, but the OTUs were classified at different taxonomic levels.

Supp. Fig 13A: Which distance matrix went into the PCoA? Weighted or unweighted unifrac? The ellipses surrounding the points suggest that a PERMANOVA or other test for clustering was performed, though this is not detailed in the methods. It would help to actually do a PERMANOVA to show the differences between the atmospheric and tank type.

Supp. Fig 13B: Not clear how phyllospheric bacteria are separated from total bacteria? were >6000 OTUs (or species based on x axis label) actually found in tank-forming species? this is suspiciously high, might be artificially inflated by a sequencing artifact.

Supp. Fig. 13CD: what tool was used to create correlations?

Supp. Fig. 14A: It's a little confusing that the bacteria are ordered differently between the mean decrease accuracy and mean decrease gin. I like the decreasing order, but it would be easier to understand if you look across and see the same genera.

Reviewer #3

(Remarks to the Author)
In this paper, the authors construct a phylogeny of air-living epiphytic plants to point to an origin and subsequent diversification ~6 million years ago in the Andes. They present a variety of 'omics datasets that point to altered metabolism, stress tolerance, and modification of roots and trichomes as adaptations underlying this major niche shift. They additionally present microbiome sequence data which suggests that nitrogen-fixing bacteria may aid in nutrient acquisition (a problem for plants that are not in contact with soil). In general I found this paper very interesting and thorough, with a well-written background and a good articulation of the specific gap that this study aims to fill. Figures are clear and well-constructed, though since the authors present so many different data types (many on different subsets of the full lineage considered in the paper), I would suggest adding an overview figure that summarizes all of the samples/analyses and the major findings. I will focus my comments on the phyllosphere microbiome analysis (my area of expertise).

Major comments
•The authors highlight two particular genera, 1174-901-12 and Sphingomonas, that are detected within the phyllospheres of tillandsioids and contain many nitrogen-fixing species. They use this result to support the claim that nitrogen-fixing bacteria help air plants to solve the problem of not being able to acquire nitrogen from the soil. This is an intriguing hypothesis but there are two significant leaps in the logic here. First, inferring function based only on genus-level information can be inaccurate, though the authors partly mitigate this concern by presenting genome-level information in the following analysis that qualitatively supports the same claim. Second, and more seriously, there is no comparison made to suggest that nitrogen-fixing bacteria are particularly enriched or otherwise playing a larger role than in other plant environments. Indeed, both of the genera that the authors choose to highlight also comprise large portions of other (non-tillandsioid) phyllosphere microbiomes. It is not clear from these data alone whether they have coevolved to take on any new or greater role alongside the niche shift of their hosts.

•I have a similar comment about the following paragraph. The authors show that nitrogenase genes are present in the T. usneoides metagenome, but with no control or comparison to suggest that nitrogen-fixing bacteria are more abundant or important in tillandsioid plants than in rooted plants. Again, nitrogenase genes have been identified in the metagenomes of other plants, so some sort of comparison is needed if the authors are claiming that they have played a particular role in adaptation here. I would also add that the presence of genes in a metagenome is not enough to conclude that they are being

actively transcribed, or even that they came from a bacterial population that was alive at the time (there is plenty of dead bacterial DNA floating around and settling on plants), but this is a common limitation of environmental microbiome studies that is hard to get around.

•I am often hesitant to suggest additional data collection/curation because I recognize the burden it can put on authors, and I am aware that the microbiome section is not the central claim of this manuscript. However, as this section currently stands, with no comparison to non-tillandsioid plants (and knowing that the genera/genes mentioned are common in other species as well) the authors' claim is not strong. I think it would not be too burdensome to download a publicly available plant microbiome dataset, ideally across multiple species, and compare whether nitrogen fixers are enriched in the tillandsioids. The authors might look to Redford et al. 2010 Environ. Microbiol., Sohrabi et al. 2023 Annu. Rev. Plant. Biol., or Smets et al. 2023 mBio as examples of phyllosphere microbiome studies with publicly available sequence data.

Minor comments
•Lines 62-63: I'm not sure of the significance of the statistic that 89% of plants are vascular plants.
•Can the authors provide more detail on the methods they used for the ancestral area reconstruction (line 137)?
•Lines 143-146, 150-152 feel like they belong in the discussion section rather than the results section.
•Lines 187-191: What makes the two species that the authors chose representative? Are they well-studied in general or was this the first study to single them out. If the latter, how were they chosen?
•Lines 557-558 and Figure S13b: What does it mean that the relative abundance of phyllospheric bacteria is higher in tank-forming than atmospheric tillandsioids? Relative abundance is normally scaled by sample, so it shouldn't be overall "higher" in one sample or set of samples than in others. Is it correct to say that the microbiomes of the tank-forming plants were composed of a smaller number of more abundant species, while the atmospheric plant microbiomes were composed of a larger number of lower-abundance species?

Minor grammatical comments:
•Line 50: "However" doesn't make sense as a transition word here, because it implies a contrast with the previous sentence.
•Line 59: "While" doesn't make sense as a transition word here, and could be removed from the sentence without losing the meaning.
•Line 122: "with lacking tanks" should be "lacking tanks" or "without tanks".
•Line 601: "when extract" should be "when extracting".
•Line 618: "fossil" should be "fossils"
•Line 621 should read "in the plant kingdom"
•Line 645-646 "No matter the rate of species variation and dispersion, or the diversity of their habitats, are extremely astonishing." This sentence is not grammatically correct, but I'm not sure how to suggest a correction because it is not clear what it is intended to say.
•Line 648: "underwent acceleration" should be "accelerated"
•Line 694 should read "function of the other organ"
•Line 727-728 should read "plenty of bacteria" (though I would suggest changing this phrase entirely to something less informal).

Reviewer #4

(Remarks to the Author)
Lyu et al. explores the evolution of the Tillandsioideae subfamily of Bromeliaceae and link life history, diversification, comparative genomic, and functional changes to processes underlying the unique biology of air plants. This paper reports over a dozen unique genome-scale analyses with probably a thousand unique individual samples that together provide significant insights into air plant evolution. However, I have some concerns. The methods are vague and in places illogical, which makes it challenging to evaluate the quality of the many analyses reported here. I have a few other comments related to the interpretation of data as well.

Major:

1. I have some serious concerns about the methods sections as many details are not clear and often nonsensical. The methods are also exclusively in the supplement. Where details are provided, programs are sometimes randomly mentioned with no context or inaccuracies in how they could be used. There is a lot of data in this paper, which is overwhelming and makes it difficult for a single reviewer to verify that all analyses are being done well. I think there is some really interesting work reported here, but more details are needed. Below I have documented some of my concerns with the methods, but this is not exhaustive:
Details on genome assembly are not clear. The authors state the draft genomes were de novo assembled using 'the de Bruijn graph', but do not specify the algorithm that was used to generate overlaps or how the actual assembly was performed. They go on to state that Hifiasm and Soapdenovo2 were employed to modify and polish the error-corrected contigs and clean reads, respectively. This is not what either of these program do. Hifiasm is an assembly algorithm and Soapdenovo2 is used to assemble Illumina short reads, and is rarely used anymore. They go on to say "The integrity, consistency, and accuracy of the assembly were evaluated using multiple bioinformatic tools such as BUSCO, BWA, Merqury, CEGMA, and samtools." I have no idea how a short read aligner like BWA or the Samtools suite could be used to assess genome quality. Without context it is also unclear how Merqury or CEGMA were used to assess genome quality.
The genome annotation is similarly vague, where programs are listed along with datasets, but how these were synthesized is not clear. For instance, five gene prediction programs are listed along with evidence, but how these data were integrated

or how the final gene models were defined is not mentioned. The identification of chromosomal rearrangements section also lists 'random' programs but provides no details about what was done.
For the Nucleotide diversity analysis, how was a vcf file generated for all 147 species? Were they aligned all to one reference genome? How could this work with the divergence across a subfamily? I have never seen this done before. I have also never seen nucleotide diversity levels calculated within a 10 bp window.
De Novo repeat annotation is described twice, in the genome annotation and de novo identification sections. How do these relate?
For RNAseq, the authors state transcriptome analyses followed the methods from Trapnell et al. (2010). This paper is 15 years old, and virtually none of the approaches and toolsets reported here could be used to analyze the data reported here, outside of the core algorithm Cufflinks. There is a paragraph about BMKMANU S1000 RNA-Seq. I was unable to find much details about this, and there are no citations in this section, but I did find a paragraph that is virtually identical to a 2023 PNAS paper on tomato.
For the random forest analysis, the authors state a random forest model was used, but do not specify what datasets were used as features, how training and testing sets were created, or what the binary classification was?
I am not sure if this reflects or a broader problem with the paper or if the authors simply need to carefully refine the methods to reflect what was actually done, and not a rough guess of what programs were used. For comparison, the comparative genomics analysis methods section provides a very detailed explanation of what was done including versions of all software and links to github pages, so I suspect more details are needed.

2. 20k gene models were annotated for V. erythrodactylon, which is very low and much lower than other Tillandsioideae. Tillandsia fasciculata and T. leiboldiana for instance have 34,886 and 38,180 gene models respectively. Also, why not compare the V. erythrodactylon and T. duratii genomes to other sequenced Tillandsioideae genomes? The T. fasciculata and T. leiboldiana genomes were published a few months ago, so the comparative analyses do not need to be redone, but these genomes should be mentioned somewhere in the paper.

3. One major difference between V. erythrodactylon and T. duratii is that T. duratii is a CAM plant while V. erythrodactylon is C3. This novel carbon concentration mechanism may be driving some of the gene family dynamics and other genome evolution, as previously found by Crego et al. Plant Cell 2024. This is not really discussed in the paper though.

4. Line 80. Numerous transitional species have been discovered across virtually all taxa, demonstrating various evolutionary traits. While Bromeliads are indeed an exciting group of plants for addressing evolutionary questions, the claim of a lack of transitional species is not justified today. This misconception, often perpetuated by creationists and other groups, is generally false and should be removed from the manuscript in my opinion.

5. It is not clear to me why root development genes would undergo positive selection in species that lack roots like the atmospheric tillandsioids. I would expect these genes to experience relaxed selection or degeneration rather than positive selection, as has been observed in parasitic plants that lose photosynthesis genes. The loss of genes involved in gravitropism and lateral root development in these species is quite interesting.

6. A paragraph seems to be duplicated in the results, lines 399-416 and lines 417-436.

7. CYP96A15 P450s are involved in cuticular wax biosynthesis. The tandem duplication of this P450 in bromeliads is not unexpected, as P450s rapidly duplicate in plant genomes, but I do not see the link for how this gene is involved in trichome formation. Instead, I would assume this gene cluster is involved in cuticle formation on the already existing trichomes. Also, the in situ hybridization results in Figure 5c are not clear to me, and I cannot see trichome specific expression. For the spatial expression, is this a new experiment or data from the root data in Figure 4?

Minor:

Line 57. It is generally not good to use the term lower plants. Similarly, what does 'advanced families' in line 65 refer to?
Line 194. What is genome completeness referring to here? BUSCO results, or assembly size compared to estimated genome size?
Line 380. What does 'raw reads blasting' mean?
Line 381. Is there a number missing for '111,73' or is the comma in the wrong place?
Line 338. I am not sure what 'SMART RNAseq is, and I could not find much via a google search. This should be defined here, as well as how it differs from typical RNAseq protocols/analyses.
Line 580. I am not a microbiome expert, but what does '1174-901-12' refer to, is this an un named genus?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
See attached review.

Reviewer #2

(Remarks to the Author)
I thank the authors for their work in revising the manuscript. Overall, most of my concerns have been sufficiently addressed. A few small points remain:

1: Thanks for the clarification about “phyllospheric” bacteria, and I understand. It would be useful to make this very clear given phyllospheric here means for atmosphere types. Adding a sentence making this point would help as many plant microbiome researchers will assume you are differentiating between root and leaf tissues.

2. Thanks for redoing the analysis with the plastids removed and adding more detail to the methods for the microbiome analyses. I apologize that I was unclear about my comment regarding rarefaction and accounting for read depth. PCoA and PERMANOVA analyses can be sensitive to the overall read number per sample. There are pros and cons to rarefying data, but if you don’t rarefy, then you should include read counts as a covariate in your PERMANOVA analysis. While LEfSe uses relative abundance, it would also help just to show that microbiomes were sampled at sufficient read depth. To do this, providing rarefaction curves as supplementary figure would help.

Reviewer #3

(Remarks to the Author)
In this revision, the authors provided additional detail on their methods, re-analyzed their microbiome data at the ASV level to the same qualitative conclusion, removed host plastid sequences, and compared the taxonomic composition of their tillandsioid microbiomes to an outgroup. The revised version of the microbiome analysis is now much stronger, and still supports their claim that nitrogen-fixing bacteria may play a particular role in plant nutrition for these species. I have only a few minor comments about remaining typos:

Line 50: change ‘have’ to ‘has’
Line 64: change ‘increasing’ to ‘expanding’
Line 99: change ‘helpful’ to ‘help’ or add ‘be’
Line 510: I don't understand the usage of the word "asymbiotic" here. Is this in reference to bacteria that do not reside in intracellular compartments? I can't tell from the methods how bacterial DNA was isolated from plants, but if there was a tissue homogenization step (as opposed to washing cells off the surface) then the bacterial sequences could very well be derived from intracellular symbionts. In any case, this seems like an unnecessary specification that could be removed from the section title.
SI Line 387: change 'arter' to 'after'

Reviewer #4

(Remarks to the Author)
the authors have addressed by previous concerns and I appreciate their detailed revisions.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily addressed all of my concerns. My thanks to them!

I do think they should determine, by inspecting maps of thermal seasonality and of the distribution of tank bromeliads (or atmospheric bromeliads), whether those kinds of epiphytes increase with seasonality, decrease with seasonality, or are dominant at an intermediate level of seasonality. Just tweaking a couple of words, based on such observations, would strengthen their ecological contribution.

Reviewer #2

(Remarks to the Author)
I thank the authors for their work in the revision in this fascinating system. My concerns have been sufficiently addressed, nothing else to add.

Reviewer #3

(Remarks to the Author)
I support publication of this manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Lyu et al. present a remarkably integrative analysis of evolution in the large bromeliad subfamily Tillandsioideae, including phylogeny, historical biogeography, climate niche evolution, genomic bases of key functional traits, and identification of N-fixing microbes associated with several epiphytic species. This paper makes several important contributions to our understanding of bromeliad evolution, and opens up new avenues of inquiry that are likely to be highly productive in the future.

However, in my opinion, a few of the authors' key conclusions are unwarranted, and the authors' fail to acknowledge some previous conclusions that parallel their own. These errors must be corrected.

Authors' response: Thank you so much for acknowledging our work. Please find our detailed responses to your comments below.

Here are my detailed comments:

1. The Abstract states that the tillandsioids arose in the Andes. This claim is based on analyses and statements on lines 137-139. In my opinion, this conclusion is not justified and is an artifact of inadequate sampling of ingroups and outgroups. Givnish et al. 2011 – not cited re historical biogeography in either the text or SI, and which included representatives of all bromeliad subfamilies – concluded that the core Tillandsioideae (which excludes the Catopsis-Glomeropitcairnia clade) arose in the Andes. It is obvious that the conclusion by Lyu et al. is an artifact of failing to include representatives of three key outgroups (bromeliad subfamilies Brocchinioideae, Lindmanioideae, and Navioideae) as well as the non-Andean ingroup Glomeropitcairnia. The two species of Catopsis they did include are restricted to Central America. A representative sampling of bromeliad ingroups and outgroups thus would not support an Andean origin for the tillandsioids. Consequently, the authors' conclusions re the region where Tillandsioideae arose are untenable. Furthermore, they fail to cite the biogeographic conclusions of Givnish et al. 2011, which bear directly on the question they attempted to address. If they correct their biogeographic analysis, they should also cite that paper as having reached the same conclusion 15 years ago.

Authors' response: Thank you for valuable comments and attention. According to Barfuss's research (2016), Tillandsioideae was categorized into core and non-core clades (as mentioned in our manuscript on lines 110-112). The Catopsis-Glomeropitcairnia clade is classified within the non-core Tillandsioideae subfamily, which aligns with our finding that Catopsis members are part of the non-core Tillandsioideae. Based on our analysis, we concluded that the core Tillandsioideae subfamily (excluding the Catopsis-Glomeropitcairnia clade) originated in the Andes, aligning with the findings presented in your published article in 2011. We have included the citation and addressed any ambiguities in the manuscript. All available evidence

supports the hypothesis that the core Tillandsioideae subfamily arose in the Andes.

2. In the SI, the authors state that four fossil dates and a secondary calibration point were used to date their phylogeny (bottom, first page). However, none of these dates are identified. Those dates must be stated explicitly!

Authors' response: Thanks for your reminder. The fossil dates were collected from the TimeTree Resource (https://timetree.org/), and then were used to estimate divergence time by MEGA 11 (Mello, 2018). We added this detailed information in the SI in lines 57-58.

Mello, B. (2018). Estimating TimeTrees with MEGA and the TimeTree Resource. Mol Biol Evol 35, 2334-2342. 10.1093/molbev/msy133.

3. Line 62: "Remarkably, epiphytes have evolved among all major lineages of land plants" – this change is necessary given the incorrect conclusions that could be drawn from the vague "all taxa of land plants" used by the authors.

Authors' response: Thanks for your suggestion. We revised this sentence in the manuscript in line 62.

4. Line 148: "Since the emergence of atmospheric bromeliads…" – Given the authors' sampling within Tillandsioideae, it does appear that there was a single origin of the atmospheric habit within Tillandsia (the only genus with atmospheric species) with only a few reversions to the tank habit. I am not an expert on Tillandsia diversity, but I found this conclusion surprising given what I know about the distribution of atmospherics in the Barfuss et al. plastid phylogeny. I do hope that Michael Barfuss has been consulted on this issue. If there is a single origin of the atmospheric habit … which is not unreasonable from an evolutionary viewpoint … that would be a big contribution of this paper. So, please confirm this point.

Authors' response: Thank you for your suggestion. We attempted to contact Dr. Barfuss twice in 2020 to consult on other questions, but did not receive any response. Nevertheless, based on our phylogenetic tree, there appears to be a single origin of the atmospheric habit from an evolutionary point. Additionally, we analyzed the plastid phylogeny of atmospheric plants as constructed by Barfuss et al., which led to the same conclusion. Given that Tillandsia is the sole genus with atmospheric species and nearly all members of Tillandsia are atmospheric, we do believe that this conclusion appears to be reasonable.

5. The discussion of tillandsioids growth forms (lines 67-85) needs to be tightened up. First, Tillandsioideae is not merely "a prominent epiphytic subfamily", it is the largest and almost all its species are epiphytic. The only other subfamily with substantial numbers of epiphytics is Bromelioideae. Second, the phrase about tillandsioids exceeding even Orchidaceae "in range" should be rewritten so as to avoid erroneous impressions by the readers – orchids have a far greater geographic distribution than bromeliads as a whole. Third, both atmospheric and tank epiphytes in Tillandsioideae have absorptive trichomes (see Benzing's books and publications re this topic. The

authors' wording implies this may not be true. Fourth, lines 76-79 require phylogenetic reconstructions of trait evolution that have never been conducted; this is plausible speculation, but speculation nonetheless. Finally, lines 79-85 imply that we're going to see analyses of transitional forms, possibly including the transition to epiphytism, given how prominently that habit features in the discussion thus far. However, the tillandsioids do not – to my knowledge – provide any system for analyzing the transition to epiphytism, given that epiphytism is ancestral to Tillandsioideae, as shown by Givnish et al. 2014 (not cited in ms.).

Authors' response: Thank you for your suggestion. Accordingly, we revised this sentence, removed the inappropriate description, and cited the relevant articles.

In addition, 'transition' here refers to changes in organs, such as the acquisition of new organs and the loss of obsolete ones, rather than transitions in habitats from terrestrial to epiphytic. In the process of plant evolution, the acquisition or loss of organs is inevitable because this process is not abrupt and is gradually evolving, thus transitional species should inevitably appear during evolution. The degeneration of roots, loss of tanks, and increased density of absorptive trichomes in Tillandsioideae plants provide excellent examples for studying organ evolution. However, we admit that further detailed research is needed on the evolution of these traits. Thank you very much for raising this question; we will delve into this question in future studies.

6. Line 86: get rid of "tachylalic" and insert "rapid".

Authors' response: We removed "tachylalic" and insert "rapid" in this sentence as suggested.

7. Line 89: get rid of "air plant" and use either of the two well-defined terms "epiphyte" or "atmospheric epiphyte" so that the readers know what is being talked about.

Authors' response: We corrected "air plant" as "epiphyte".

8. Line 95: One of the most important aspects of this paper is unannounced in the abstract AND here – namely, that the new phylogeny presented is based on (largely) nuclear transcriptomic sequences. Please remedy this! This novel basis, however, requires some statements in the Results and prep in the SI methods what broadscale did erences the authors see between their nuclear phylogeny and earlier, even more thoroughly sampled phylogenies based on plastid genes by Barfuss and his colleagues.

Authors' response: Thanks for your valuable suggestion. We added this important work we did in the abstract line 30, and some statements in the Results lines 103-105, discussion lines 602-612 and SI methods lines 31-56.

9. Lines 104-110: Apparently, based on the elliptical statements in the SI, the authors applied maximum likelihood on the concatenated data to obtain their nuclear phylogeny. Fine. BUT there are several shortcomings: (a) I do not see bootstrap support values anywhere; that must be remedied, perhaps in the SI. As it is, we cannot assess the extent to which the authors' findings are definitive. (b) It is standard procedure to present (and, often, to use) ASTRAL analyses of nuclear data, to move from gene trees to species

trees – this should include some analyses of discordance. (c) Some statement MUST be made about how the Lyu et al. nuclear phylogeny diders from that advanced by Barfuss et al. 2012 and 2016.

Authors' response: Thanks for your valuable suggestion. (a) We have included the bootstrap values in Supplementary Fig. 2. (b) According to your suggestion, we conducted ASTRAL analyses and included this comparison in Supplementary Fig. 2. (c) In the revised manuscript lines 602-612, we have included further discussion on comparing our nuclear phylogeny with the plasmid phylogeny presented by Barfuss et al.

10. Lines 160-162: Specify the actual nature of the association of each growth form with each environmental variable. For example, is the atmospheric habit associated with colder or warmer temperatures?

Authors' response: Thanks for your comments. It might be a misunderstanding about the interpretation of this analysis. While climate change influences species evolution, different species respond differently to environmental factors due to their distinct genomic backgrounds. In essence, the primary environmental drivers of species evolution vary among species. Here, we examine the principal environmental factors that have influenced the evolution and distribution of two types of tillandsioids, rather than asserting a direct relationship between their habitats and these environmental factors. For instance, we observed that the distribution of atmospheric tillandsioids is predominantly influenced by the mean temperature of the coldest quarter. Similarly, this factor may also drive the distribution of species from other genera, families, or orders that exhibit different habits.

11. Lines 309-311: The authors should cite Givnish et al. 1997 (in Givnish & Sytsma, Molecular Evolution and Adaptive Radiation), who over 25 years ago identified the connection of the tank habit to soft, easily conformable leaves and broad leaves (e.g., "the tank habit, perhaps because the latter requires relatively soft, broad leaves that conform tightly to each other …").

Authors' response: Thank you for bringing this to our attention. We cited this paper in the manuscript line 304.

12. Lines 321-323: The authors should cite Benzing 2000, at least, to support this statement.

Authors' response: Thank you for bringing this to our attention. We added this citation in the manuscript line 316.

13. Lines 459-461: The apparent claim that all trichomes have a waxy cuticle is supported, as far as I can see, by studies outside Bromeliaceae. Furthermore, the death of trichomes exposed to desiccation in Brocchinia (Givnish et al. 1997) argues against this as a universal rule. Perhaps it is my ignorance, but I have seen studies of trichomes in tank epiphytes in Tillandsioideae and Bromelioideae to know if their live trichomes are covered with wax. I raise this question because the presence of a waxy cuticle might

interfere with water or nutrient uptake by trichomes.

Authors' response: Thanks for your comments. Here we investigated the genes potentially involved in trichome waxy cuticle biosynthesis in bromeliaceae plants, with differentiation observed between terrestrial and epiphytic bromeliads. However, we cannot definitively conclude whether this differentiation relates to the water absorption function of the trichomes. We hypothesize that the presence of a waxy cuticle may serve to prevent water loss in the plant. Regarding whether the waxy cuticle could affect water or nutrient uptake by trichomes, our speculation leans towards no. This is because the primary mechanism of water absorption in tillandsioid trichomes does not involve cell membrane permeability but rather relies on the unique trichome structure described by Raux et al. (2020).

Raux, P.S., Gravelle, S. & Dumais, J. Design of a unidirectional water valve in Tillandsia. Nat Commun 11, 396 (2020). https://doi.org/10.1038/s41467-019-14236-5

14. Lines 536 and following: This section is, to me, one of the most interesting contributions in the paper. However, the authors should cite Givnish et al. 1997 for the presence of N-fixing cyanobacterial in the tanks of certain Brocchinia species.

Authors' response: Thank you for bringing this to our attention. We added this citation in the manuscript line 525.

15. Line 626: Again, origin of tillandsioids in the Andes is NOT supported!

Authors' response: Thanks for your valuable comment. We revised this conclusion from "tillandsioids originated from the Andes" to "core tillandsioids originated from the Andes" in the manuscript line 613.

16. Discussion: Here, and in the intro, the authors should cite Givnish et al. 2014, who found that epiphytism in Bromeliaceae always arose in tank-forming species. Here, and in the intro, the authors should also cite Givnish et al. 2014 and Givnish et al. 2015 (Proc R Soc B) for the general finding that epiphytism accelerates species diversification, as well as their interpretation of why that occurs. I waive my right to anonymity as a reviewer.

Authors' response: Thank you for bringing this to our attention. We cited these two articles in the manuscript lines 636.

Sincerely yours,

Thomas J. Givnish

Henry Allan Gleason Professor of Botany

Wilhelm Hofmeister Professor of Botany

University of Wisconsin-Madison

Reviewer #2 (Remarks to the Author):

This manuscript seeks to understand innovations in the evolution of epiphytic plants. By combining multiple 'omics technologies with a highly resolved phylogenetic tree, the authors provide insight into the evolutionary genetics involved in important traits that enabled the transition from soil to air lifestyle. I was asked to comment on the microbiome analyses, and so my review is focused on those sections.

Overall, there is insufficient detail provided by the authors to evaluate the microbiome analyses.

Authors' response: Thank you for acknowledging our work. Please find our responses to your comments below.

with the claim that the relative abundance of "phyllospheric" differ between tank and atmospheric types (lines 557-558). It seems like the bacterial community is being partitioned into a phyllospheric and non-phyllospheric community. As leaf tissue was used to generate the 16S data, then all OTUs are from the phyllosphere. So how the microbiome is being separated into these two components needs additional justification and detail.

Authors' response: We are sorry for any misunderstanding caused by our initial unclear wording in the analysis interpretation. The terms 'tank' and 'atmospheric' types pertain to plant classifications, not bacterial communities. Therefore, we did not differentiate the bacterial community into phyllospheric and non-phyllospheric categories; all bacteria analyzed are from the phyllosphere. We have revised this statement to improve clarity and avoid further confusion in manuscript lines 532-536.

The network analysis (supp. fig. 13 and result lines 561-565) does not say anything about "dominant genera".

Authors' response: Thank you for bringing this to our attention. We have removed the incorrect figure citation (supp. fig. 13) and retained only the citation for Fig. 7 in this section.

Finally, the conclusion that nitrogen fixing bacteria are an important aspect of tillandsioids biology that underlie adaptation to the air lifestyle is unsupported. As the authors state, these nitrogen fixing bacteria, like Sphingomonas, are commonly found on diverse plants, and so it's not clear if there is some sort of special enrichment for nitrogen fixing bacteria; this would really need additional work. Only one species of tank type (I think the reviewer referred to atmospheric type) was used in the metagenome analysis, and so connecting these results further to the distinction of tank and atmospheric is difficult. You could probably mine the Pacbio HiFi genomes for more detailed metagenomic information to broaden the comparison.

Authors' response: Thanks for your suggestion. We fully agree with you that further investigation is needed in the future to determine whether there is any specific enrichment for nitrogen-fixing bacteria and to explore the interaction mechanisms between these bacteria and plants. In fact, in addition to the atmospheric type, we also

assembled a tank-type metagenome (V77). We only identified nitrogenase genes, required for nitrogen fixation, in the metagenome of atmospheric-type plant TU (Supplementary Fig. 14&15). While the presence of these genes in the metagenome of the plant's phyllosphere indicates the presence of nitrogen fixing bacteria, we cannot draw any conclusions from the absence of such genes in the other metagenomic samples (absence of proof is not proof of absence).These results could either mean that nitrogen fixation associated genes truly do not exist in tank-type plants' microbiome or that the quantity of microbes possessing these genes in the sample was too low to be represented in the metagenome. As our focus was to confirm the presence of nitrogenase genes in leaf metagenomes of atmospheric type tillandsioids, we did not discuss the tank-type metagenome in depth, but data for the V77 metagenome has been deposited in the NCBI database.

Regarding your suggestion to mine the PacBio HiFi genomes for more detailed metagenomic information, we really regret that this analysis would not lead to the envisioned results. At the initiation of this project, when attempting to extract DNA from tillandsioids for genome sequencing and assembly, we encountered significant challenges in obtaining clean DNA from the leaves of atmospheric tillandsioids due to frequent contamination by microbial DNA. Despite trying various methods of washing and cleaning, we were unsuccessful. The microorganisms attached to the epidermis could not be effectively removed; when the plant's leaf surface contacted the liquid, the wing cells of the trichome adhered to the leaf epidermis, preventing the removal of a substantial number of microorganisms beneath these wing cells. Ultimately, we resorted to blade cutting to remove both the trichomes, epidermis and its underlying tissue, extracting pure DNA only from the remaining internal leaf tissue for genome assembly. Therefore, the current assembled PacBio HiFi genome lacks genetic information about these phyllosphere microorganisms. Hope you could understand this situation in peculiar DNA extraction for tillandsioids genome assembly.

Another key point of concern is that it's not clear how the growing environment is controlled for in the microbiome samples. I recognize the difficulty of growing this different plant species, but whether the plants were grown at the same time or under the same environmental growth conditions could confound the microbiome analysis. More clarity is needed.

Authors' response: Approximately half of the plants were cultivated in the greenhouse at the Shanghai Chenshan Botanical Garden, with the remaining half grown in the greenhouse at the Bromeliads Research Center of Zhejiang Academy of Agricultural Sciences in China. The growth conditions in both greenhouses were quite similar. Samples were collected from both locations within a day of each other. Despite the plants being cultivated in different environments, we observed similar compositions of phyllosphere microbes among plants of the same species. It is really interesting for the host species specificity for colonizing specific phyllosphere microbes that is worth to be elucidated about its mechanism. Additional details regarding sampling locations are provided in Supplemental Table 8.

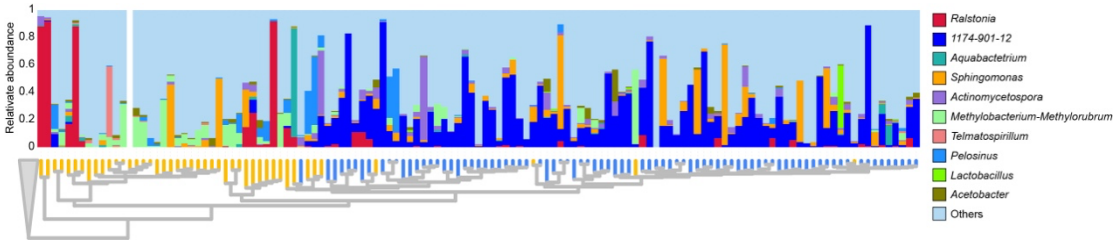
I next organize my comments to provide feedback based on methods and figures.

For 16S rRNA profiling, it is unusual to see data presented as OTUs. Most microbes studies have used ASVs for at least the past 5 years. There is nothing inherently wrong with using OTUs, but this rationale should be explained.

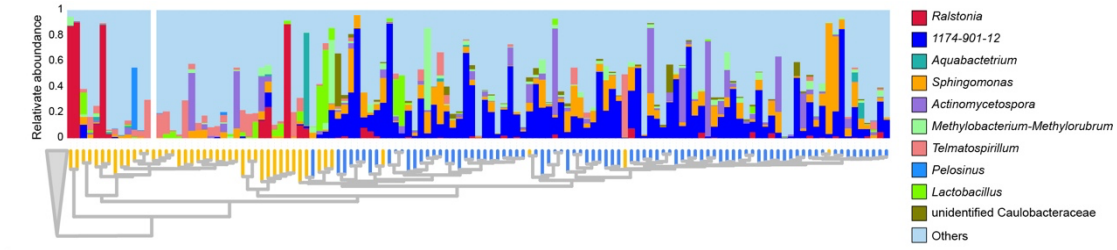
Authors' response: Thanks for your comments. Surely, ASVs offer more precise measurements of sequence variation. However, for investigating broad-scale ecological diversity, OTUs are deemed more suitable, as indicated by Glassman and Martiny (2018). Given the relatively large population size we studied, we employed the OTU clustering method in our research. Here, we also delineated microbial taxa using ASVs. We found both methods yielded similar results. The following figures display the relative abundance of phyllospheric bacterial communities at the genus level using OTUs and ASVs, respectively. The top 10 genera are similar.

Glassman, S. I., & Martiny, J. B. (2018). Broadscale ecological patterns are robust to use of exact sequence variants versus operational taxonomic units. MSphere, 3(4), 10-1128.

Top-10 genera by OUT:



Top-10 genera by AVS:



Variation in read depth needs to be clarified. Rarefaction curves are needed to show that communities were sufficiently sampled. Given that V3-V4 region was used, chloroplasts often comprise a substantial amount of reads, without using peptide nucleic acid clamps. Presumably plastid sequences were removed from analysis, but it is not clear what the authors did (and rarefaction should be done after chloroplast removal). I can see that "f__mitochondria" were identified as differentially abundant in the Fig. 7C cladogram, which suggests that this kind of filtering was not applied. Plastids are normally removed from 16s analysis.

Authors' response: Thank you for highlighting this issue. We have filtered data before performing the following analysis as described in the SI methods lines 382-383. Here,

we reanalyzed all results by using filtered data (plastids were removed after annotation), and the updated results are presented in Figure 7c.

Whether or not the data was rarefied before PCoA or LEfSe analysis is important because variation in read depth across samples needs to be accounted for in the statistical model. Similarly, it matters if the rarefaction curve has reached saturation. This needs to be clarified.

Authors' response: Thank you for bringing this to our attention. We re-performed the PCoA analysis using filtered data (plastids were removed), and the updated results are presented in Supplementary Fig. 15a.

Furthermore, for the OTU differential abundance, it is normal that some pre-processing/filtering is applied to the data set (i.e., include only OTUs that occur across a majority of samples across categories). Again, it's important to know if the data was rarefied or not before differential abundance analysis and how this was handled. Similarly, there is no method described that generated the network analysis in Supp. Fig. 13.

Authors' response: Thank you for bringing this to our attention. We re-performed the abundance analysis using filtered data (plastids were removed), and the updated results are presented in Supplementary Fig. 15b. Additionally, we included the methods for the network analysis in SI methods lines 402-410.

A similar lack of important methodological detail also exists for the metagenomes.

Authors' response: Thank you for bringing this to our attention. We have included the methods for the assembly and annotation of metagenomme in the SI lines 421-464.

Some specific comments on the microbiome figures:
Fig. 7A: SEM images are not super instructive as which plant is not labelled, and while the arrows point to "microorganisms", which could include both fungi and bacteria, the data are only on bacteria. I did not find this figure to be particularly useful.

Authors' response: Thanks for your comments. Our original objective was to visually illustrate the presence of microorganisms on the leaf surfaces of tillandsioids. As our focus was solely on bacteria, the images exclusively depict bacterial presence. We have included the plant names in the SEM images.

Fig. 7B: "others' category makes up most of a lot of samples. what is in the others?

Authors' response: Figure 7B displays only the top 10 genera by relative abundance. 'Others' refers to the sum of all genera outside of the top 10.

Fig. 7C: I don't really understand what this cladogram is supposed to show. I'm guessing the non-highlighted nodes are not differentially abundant between tank and atmosphere type? It just isn't intuitive about how to relate the cladogram to differential abundance. The heat map in supp. fig. 14 is maybe more intuitive? I also don't understand what "phylogenetic distribution from phylum to genus of the microbiota"

means here. I'm confused because the tree was based on OTU sequences, but the OTUs were classified at different taxonomic levels.

Authors' response: We are sorry for any confusion caused by the lack of captions that may have made these results difficult for you to interpret. This cladogram aims to show the abundance differences of phyllospheric bacteria enriched in two types of plants at various taxonomic levels. In the phylogenetic tree, the concentric circles radiating outward represent taxonomic levels from phylum to genus (or species). Each small circle at different taxonomic levels represents a classification at that level, with the diameter of each circle proportional to its relative abundance. Species with no significant differences are uniformly colored light blue. Species (or genera, or families, or orders, or phylum) with differences are colored according to their groups; orange nodes represent bacterial groups that play important roles in tank-forming plants, while dark blue nodes represent those in atmospheric-type plants. Species (or genera, or families, or orders, or phylum) names represented by letters in the figure are explained in the legend at the bottom. We included the captions in the figure legends lines 1188-1196.

We believe this LEFSE cladogram is more intuitive than heat map. Because this cladogram cannot only show the abundance differences between the two types of tillandsioids at different taxonomic levels, but also reveals the relationship between these different species (or genera, or families, or orders, or phylum) in the bacterial evolutionary branch as well.

Supp. Fig 13A: Which distance matrix went into the PCoA? Weighted or unweighted unifrac? The ellipses surrounding the points suggest that a PERMANOVA or other test for clustering was performed, though this is not detailed in the methods. It would help to actually do a PERMANOVA to show the differences between the atmospheric and tank type.

Authors' response: Binary jaccard distance matrix was used in the PCoA analysis. We performed PERMANOVA analysis (also known as ADONIS analysis) using the ADONIS function from the R vegan package (https://cran.r-project.org/web/packages/vegan/index.html). The two clustering ellipses were computed with the ggplot2 function stat_ellipse, applying a 95% confidence level. We included the methods details in the SI lines 392-397 and updated the Supplementary figure 15a.

Supp. Fig 13B: Not clear how phyllospheric bacteria are separated from total bacteria? were >6000 OTUs (or species based on x axis label) actually found in tank-forming species? this is suspiciously high, might be artificially inflated by a sequencing artifact.

Authors' response: We did not separate the bacterial community into phyllospheric and non-phyllospheric categories; all bacteria analyzed are from the phyllosphere. The previous high number of OTUs was due to the unfiltered data we used and the combination of all species into two groups. According to another reviewer's comment, we noticed it is inaccurate to state that the microbiomes of the tank-forming plants

consisted of a smaller number of more abundant species. We have reanalyzed the relative abundance of all species rather than combined them into two groups using filtered data (plastids were removed). The updated result has been reflected in Supplementary Fig. S15b, and we have revised the corresponding description in the manuscript lines 532-536.

Supp. Fig. 13CD: what tool was used to create correlations?

Authors' response: We included the methods for the network analysis in SI methods lines 402-410.

Supp. Fig. 14A: It's a little confusing that the bacteria are ordered differently between the mean decrease accuracy and mean decrease gin. I like the decreasing order, but it would be easier to understand if you look across and see the same genera.

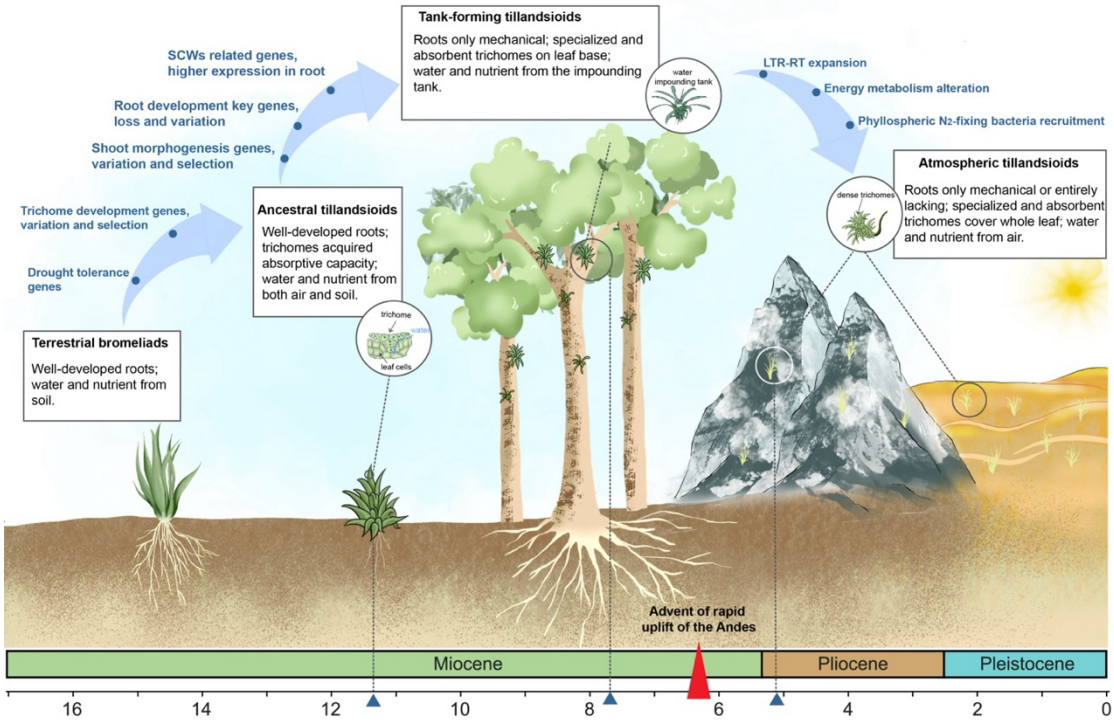
Authors' response: This figure shows the Random Forest results of the top-30 genera by Mean Decrease Accuracy and Mean Decrease Gin, respectively. Mean Decrease Accuracy measures the reduction in prediction accuracy of a random forest when the values of a variable are turned into random numbers. A larger value indicates greater importance of the variable. Mean Decrease Gini calculates the impact of each variable on the heterogeneity of observation values at each node of a classification tree using the Gini index, thereby comparing the importance of variables. A larger value indicates greater importance of the variable.

Due to the differences between the two calculation methods, the top-30 important genera calculated by the two methods are not completely the same, so the left and right charts cannot correspond one by one.

Reviewer #3 (Remarks to the Author):

In this paper, the authors construct a phylogeny of air-living epiphytic plants to point to an origin and subsequent diversification ~6 million years ago in the Andes. They present a variety of 'omics datasets that point to altered metabolism, stress tolerance, and modification of roots and trichomes as adaptations underlying this major niche shift. They additionally present microbiome sequence data which suggests that nitrogen-fixing bacteria may aid in nutrient acquisition (a problem for plants that are not in contact with soil). In general, I found this paper very interesting and thorough, with a well-written background and a good articulation of the specific gap that this study aims to fill. Figures are clear and well-constructed, though since the authors present so many different data types (many on different subsets of the full lineage considered in the paper), I would suggest adding an overview figure that summarizes all of the samples/analyses and the major findings. I will focus my comments on the phyllosphere microbiome analysis (my area of expertise).

Authors' response: Thank you for acknowledging our work and your valuable suggestion. We have prepared an overview figure summarizing all major findings, which is designated as Figure 8 in the manuscript.



Major comments

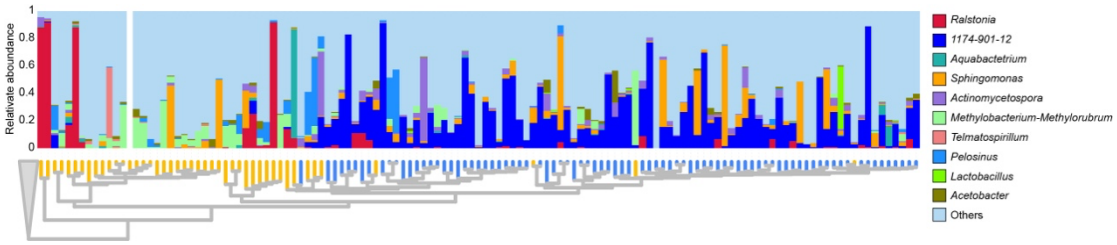
•The authors highlight two particular genera, 1174-901-12 and Sphingomonas, that are detected within the phyllospheres of tillandsioids and contain many nitrogen-fixing species. They use this result to support the claim that nitrogen-fixing bacteria help air plants to solve the problem of not being able to acquire nitrogen from the soil. This is an intriguing hypothesis but there are two significant leaps in the logic here.

First, inferring function based only on genus-level information can be inaccurate, though the authors partly mitigate this concern by presenting genome-level information in the following analysis that qualitatively supports the same claim.

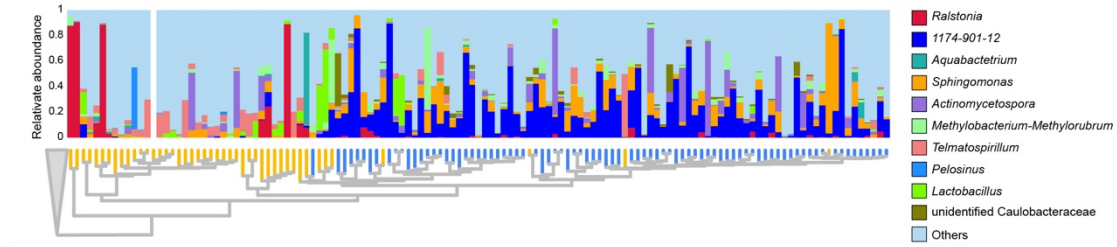
Authors' response: In fact, we have attempted to conduct the analysis from the species level. However, probably because the precision of the 16S sequencing identification is not high enough, or the phyllospheric bacteria species on the tillandsioid plants have not been accurately identified so far, our results at the species level were not ideal. For instance, when we analyzed the top ten most significant genera, we identified *1174-901-12* as potentially the most crucial genus for atmospheric-type plants. However, due to the limited research on the species of this genus, the current bacterial analysis databases do not contain sufficient information on related species of this genus, resulting in our inability to identify related species at the species level.

The following figures show the results of relative abundance analysis conducted at the species and genus levels using both OUT and ASV methods, respectively. It can be observed that the results are similar when analyzing at the genus level using OUT and ASV methods. However, there are significant differences at the species level analysis, and many important species from certain genera are missing.

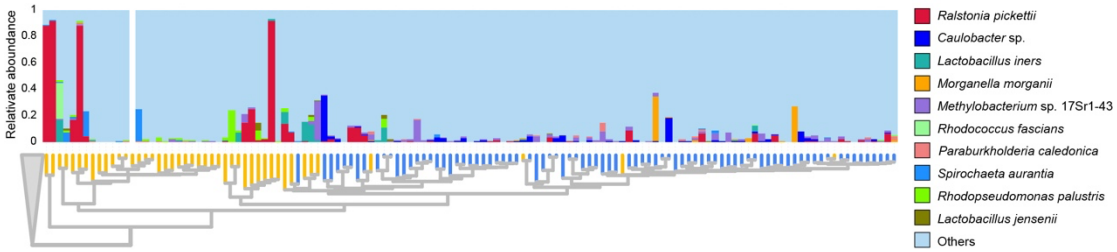
Top-10 genera by OUT:



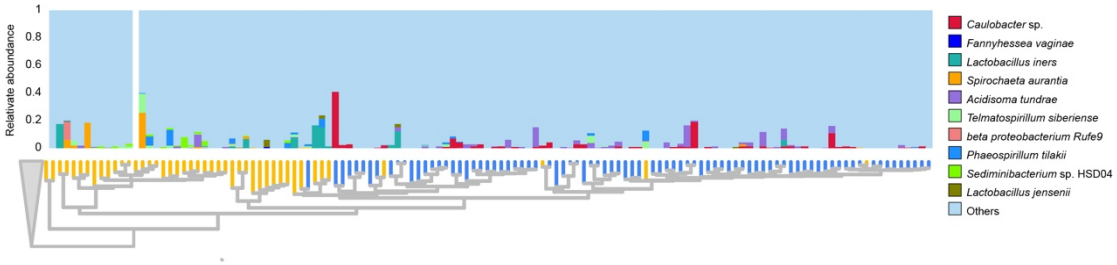
Top-10 genera by AVS:



Top-10 species by OUT:



Top-10 species by AVS:



Second, and more seriously, there is no comparison made to suggest that nitrogen-fixing bacteria are particularly enriched or otherwise playing a larger role than in other plant environments. Indeed, both of the genera that the authors choose to highlight also comprise large portions of other (non-tillandsioid) phyllosphere microbiomes. It is not clear from these data alone whether they have coevolved to take on any new or greater role alongside the niche shift of their hosts.

Authors' response: Thanks for your valuable comments. Indeed, we cannot provide a definitive conclusion on the specific enrichment of these nitrogen-fixing bacteria on tillandsioid leaves. However, these nitrogen-fixing bacteria do constitute dominant populations among the phyllosphere baterial communities of atmospheric type tillandsioids, especially genera *1174-901-12* and *Sphingomonas*. In comparison to tank-forming-type tillandsioids, bacteria from genera *1174-901-12* and *Sphingomonas*

dominate the phyllosphere bacterial community of atmospheric-type tillandsioids. What we emphasize here is that bacteria from these two genera may play a significant role in atmospheric-type plants. The term 'co-evolution' is indeed inappropriate here, and we have revised this description in the manuscript.

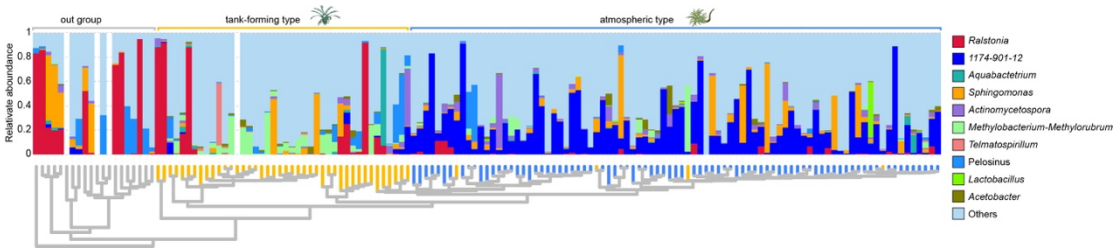
I have a similar comment about the following paragraph. The authors show that nitrogenase genes are present in the *T. usneoides* metagenome, but with no control or comparison to suggest that nitrogen-fixing bacteria are more abundant or important in tillandsioid plants than in rooted plants. Again, nitrogenase genes have been identified in the metagenomes of other plants, so some sort of comparison is needed if the authors are claiming that they have played a particular role in adaptation here. I would also add that the presence of genes in a metagenome is not enough to conclude that they are being actively transcribed, or even that they came from a bacterial population that was alive at the time (there is plenty of dead bacterial DNA floating around and settling on plants), but this is a common limitation of environmental microbiome studies that is hard to get around.

Authors' response: In fact, in addition to the atmospheric type, we also assembled a tank-type metagenome (V77). We only identified nitrogenase genes, required for nitrogen fixation, in the metagenome of atmospheric-type plant TU (Supplementary Fig. 14&15). While the presence of these genes in the metagenome of the plant's phyllosphere indicates the presence of nitrogen fixing bacteria, we cannot draw any conclusions from the absence of such genes in the other metagenomic samples (absence of proof is not proof of absence).These results could either mean that nitrogen fixation associated genes truly do not exist in tank-type plants' microbiome or that the quantity of microbes possessing these genes in the sample was too low to be represented in the metagenome. As our focus was to confirm the presence of nitrogenase genes in leaf metagenomes of atmospheric type tillandsioids, we did not discuss the tank-type metagenome in depth, but data for the V77 metagenome has been deposited in the NCBI database.

I am often hesitant to suggest additional data collection/curation because I recognize the burden it can put on authors, and I am aware that the microbiome section is not the central claim of this manuscript. However, as this section currently stands, with no comparison to non-tillandsioid plants (and knowing that the genera/genes mentioned are common in other species as well) the authors' claim is not strong. I think it would not be too burdensome to download a publicly available plant microbiome dataset, ideally across multiple species, and compare whether nitrogen fixers are enriched in the tillandsioids. The authors might look to Redford et al. 2010 Environ. Microbiol., Sohrabi et al. 2023 Annu. Rev. Plant. Biol., or Smets et al. 2023 mBio as examples of phyllosphere microbiome studies with publicly available sequence data.

Authors' response: Thanks for your suggestion. To make comparisons, we conducted 16S sequencing and analysis of phyllosphere bacterial communities of outgroup species (samples of these species were collected and sequenced at the same time as Tillandsioids species). It is obvious that the enriched phyllosphere bacterial

communities of outgroup plants are distinctly different from tank-forming or atmospheric-type tillandsioids plants. This result suggests that the significant enrichment of these nitrogen-fixing bacteria may be closely related to species specificity. We have updated these results in Fig. 7b.



Minor comments

•Lines 62-63: I'm not sure of the significance of the statistic that 89% of plants are vascular plants.

Authors' response: We removed this sentence from the manuscript.

•Can the authors provide more detail on the methods they used for the ancestral area reconstruction (line 137)?

Authors' response: We provide the detail on the methods using for the ancestral area reconstruction in the manuscript lines 90-104.

•Lines 143-146, 150-152 feel like they belong in the discussion section rather than the results section.

Authors' response: Thanks for your suggestion, we have removed these lines from the results section and included them in the discussion section.

•Lines 187-191: What makes the two species that the authors chose representative? Are they well-studied in general or was this the first study to single them out. If the latter, how were they chosen?

Authors' response: They are two relatively well-known ornamental plants. Additionally, the leaves of *T. duratii* are thicker, which makes it easier for us to cut away the leaf epidermal tissue to obtain clean DNA for genome assembly. Because the leaf surface microbiota of atmospheric type tillandsioids (which would contaminate the plant's DNA during extraction) cannot be removed cleanly, they can only be removed by using a blade. Thicker leaves are easier to handle according to our experimental experiences.

•Lines 557-558 and Figure S13b: What does it mean that the relative abundance of phyllospheric bacteria is higher in tank-forming than atmospheric tillandsioids? Relative abundance is normally scaled by sample, so it shouldn't be overall "higher" in one sample or set of samples than in others. Is it correct to say that the microbiomes of the tank-forming plants were composed of a smaller number of more abundant species,

while the atmospheric plant microbiomes were composed of a larger number of lower-abundance species?

Authors' response: Thank you for bringing this to our attention. We agree that it is inaccurate to state that the microbiomes of the tank-forming plants consisted of a smaller number of more abundant species. We have reanalyzed the relative abundance of all species rather than combined them into two groups. The updated results have been reflected in Supplementary Fig. S15b, and we have revised the corresponding description in the manuscript at lines 532-536.

Minor grammatical comments:
•Line 50: "However" doesn't make sense as a transition word here, because it implies a contrast with the previous sentence.

Authors' response: We removed this word from the sentence.
•Line 59: "While" doesn't make sense as a transition word here, and could be removed from the sentence without losing the meaning.

Authors' response: We removed this word from the sentence.
•Line 122: "with lacking tanks" should be "lacking tanks" or "without tanks".

Authors' response: We corrected "with lacking tanks" to "lacking tanks".
•Line 601: "when extract" should be "when extracting".

Authors' response: We corrected "extract" to "extracting".
•Line 618: "fossil" should be "fossils"

Authors' response: We corrected "fossil" to "fossils".
•Line 621 should read "in the plant kingdom"

Authors' response: We corrected "in plant kingdom" to "in the plant kingdom".

•Line 645-646 "No matter the rate of species variation and dispersion, or the diversity of their habitats, are extremely astonishing." This sentence is not grammatically correct, but I'm not sure how to suggest a correction because it is not clear what it is intended to say.

Authors' response: What we mean here is that tillandsioids plants evolve and spread very rapidly. It might be that we omitted the word 'tillandsioids' which caused confusion in the sentence. We revised this sentence to "The tillandsioids plants are extraordinarily remarkable in both the speed of their species variation and dispersion, as well as in the diversity of their habitats."

•Line 648: "underwent acceleration" should be "accelerated"

Authors' response: We corrected "underwent acceleration" to "accelerated".

•Line 694 should read "function of the other organ"

Authors' response: We corrected "another" to "the other".

•Line 727-728 should read “plenty of bacteria” (though I would suggest changing this phrase entirely to something less informal).

Authors’ response: We corrected “plenty” to “plenty of”.

Reviewer #4 (Remarks to the Author):

Lyu et al. explores the evolution of the Tillandsioideae subfamily of Bromeliaceae and link life history, diversification, comparative genomic, and functional changes to processes underlying the unique biology of air plants. This paper reports over a dozen unique genome-scale analyses with probably a thousand unique individual samples that together provide significant insights into air plant evolution. However, I have some concerns. The methods are vague and in places illogical, which makes it challenging to evaluate the quality of the many analyses reported here. I have a few other comments related to the interpretation of data as well.

Authors’ response: Thank you for acknowledging our work. Please find our responses to your comments below.

Major:

1. I have some serious concerns about the methods sections as many details are not clear and often nonsensical. The methods are also exclusively in the supplement. Where details are provided, programs are sometimes randomly mentioned with no context or inaccuracies in how they could be used. There is a lot of data in this paper, which is overwhelming and makes it difficult for a single reviewer to verify that all analyses are being done well. I think there is some really interesting work reported here, but more details are needed. Below I have documented some of my concerns with the methods, but this is not exhaustive: Details on genome assembly are not clear. The authors state the draft genomes were de novo assembled using ‘the de Bruijn graph’, but do not specify the algorithm that was used to generate overlaps or how the actual assembly was performed. They go on to state that Hifiasm and Soapdenovo2 were employed to modify and polish the error-corrected contigs and clean reads, respectively. This is not what either of these program do. Hifiasm is an assembly algorithm and Soapdenovo2 is used to assemble Illumina short reads, and is rarely used anymore. They go on to say “The integrity, consistency, and accuracy of the assembly were evaluated using multiple bioinformatic tools such as BUSCO, BWA, Merqury, CEGMA, and samtools.” I have no idea how a short read aligner like BWA or the Samtools suite could be used to assess genome quality. Without context it is also unclear how Merqury or CEGMA were used to assess genome quality. The genome annotation is similarly vague, where programs are listed along with datasets, but how these were synthesized is not clear. For instance, five gene prediction programs are listed along with evidence, but how these data were integrated or how the final gene models were defined is not mentioned. The identification of chromosomal rearrangements section also lists ‘random’ programs but provides no details about what was done.

Authors' response: Sorry for the incomplete and overly simplified description of the genome assembly and annotation method. We have revised the Methods section to include a more detailed and comprehensive description. Please find the detailed methods in the SI lines 113-215.

For the Nucleotide diversity analysis, how was a vcf file generated for all 147 species? Were they aligned all to one reference genome? How could this work with the divergence across a subfamily? I have never seen this done before. I have also never seen nucleotide diversity levels calculated within a 10 bp window.

Authors' response: This analysis is to identify the nucleotide diversity of the target gene (CDS region) among different bromeliad species. The details of the analysis procedure were included in the revised SI lines 239-253.

We know that this analysis method is usually applied within different individuals of the same species, but also can work for very closely related species. Here, we are attempting to apply this analysis method across different species within the same family. Variation in species traits is caused by genetic variation, and the association between these traits and gene sequences is applicable not only within different individuals of the same species but also across a broader range of species. When analyzing traits such as tank formation or trichome density within this family, distinct clades with significant differences in traits exist among species, indicating that the initiation or evolution of traits is likely driven by sequence variation and selection. Therefore, we analyzed the sequence differences of candidate genes potentially controlling the formation of these phenotypes. Typically, the window size for Fst calculation is 500 kb with a step size of 50 kb. The larger the Fst region, the longer the step size set. However, in this case, since we are using single-gene CDS with shorter sequences, we have chosen a smaller step size.

De Novo repeat annotation is described twice, in the genome annotation and de novo identification sections. How do these relate?

Authors' response: They are different methods. During genome annotation, identified repetitive sequences may appear as partial fragments of repeat sequences. LTR_FINDER and RepeatModeler software annotate any sequence they classify as a repeat, including those with incomplete structures, based on their specific criteria for repeat classification. Additionally, some repeats are identified through homology-based annotation methods. During LTR analysis, our goal is to identify repeat sequences with complete structures. To achieve this, we perform de novo annotation of repeat sequences using tRNAscan-SE and then annotate their structures using ltrdigest, thereby identifying repeat sequences with complete structures. We included this explanation in the SI lines 255-256

For RNAseq, the authors state transcriptome analyses followed the methods from Trapnell et al. (2010). This paper is 15 years old, and virtually none of the approaches and toolsets reported here could be used to analyze the data reported here, outside of the core algorithm Cufflinks. There is a paragraph about BMKMANU S1000 RNA-

Seq. I was unable to find much details about this, and there are no citations in this section, but I did find a paragraph that is virtually identical to a 2023 PNAS paper on tomato.

Authors' response: Sorry for the incorrect methods for UMI RNA-seq, and incomplete and overly simplified description of the BMKMANU S1000 RNA-Seq method. We have revised the Methods section to include a more detailed and comprehensive description. Please find the detailed methods in the SI lines 336-370.

For the random forest analysis, the authors state a random forest model was used, but do not specify what datasets were used as features, how training and testing sets were created, or what the binary classification was?

Authors' response: Thanks for bring this to our attention. We provided more details about the methods for the random forest analysis in the SI lines 319-339.

I am not sure if this reflects or a broader problem with the paper or if the authors simply need to carefully refine the methods to reflect what was actually done, and not a rough guess of what programs were used. For comparison, the comparative genomics analysis methods section provides a very detailed explanation of what was done including versions of all software and links to github pages, so I suspect more details are needed.

Authors' response: Thank you for your valuable suggestion. We have included additional details about all the methods we used like the comparative genomics analysis methods section in the revised SI.

2. 20k gene models were annotated for V. erythrodactylon, which is very low and much lower than other Tillandsioideae. Tillandsia fasciculata and T. leiboldiana for instance have 34,886 and 38,180 gene models respectively. Also, why not compare the V. erythrodactylon and T. duratii genomes to other sequenced Tillandsioideae genomes? The T. fasciculata and T. leiboldiana genomes were published a few months ago, so the comparative analyses do not need to be redone, but these genomes should be mentioned somewhere in the paper.

Authors' response: Thank you for your suggestion. We compared the difference and cited this article in the manuscript lines 198-204.

3. One major difference between V. erythrodactylon and T. duratii is that T. duratii is a CAM plant while V. erythrodactylon is C3. This novel carbon concentration mechanism may be driving some of the gene family dynamics and other genome evolution, as previously found by Crego et al. Plant Cell 2024. This is not really discussed in the paper though.

Authors' response: Since our research primarily focused on the evolution of this subfamily and the development of root, trichome, and tank traits enabling adaptation to diverse aerial habitats, we had not previously explored the carbon concentration mechanism. Following your suggestion, we have included a discussion about carbon concentration mechanism in tillandsioids in this revised manuscript lines 658-666.

4. Line 80. Numerous transitional species have been discovered across virtually all taxa, demonstrating various evolutionary traits. While Bromeliads are indeed an exciting group of plants for addressing evolutionary questions, the claim of a lack of transitional species is not justified today. This misconception, often perpetuated by creationists and other groups, is generally false and should be removed from the manuscript in my opinion.

Authors' response: According to your suggestion, we removed this sentence from the manuscript.

5. It is not clear to me why root development genes would undergo positive selection in species that lack roots like the atmospheric tillandsioids. I would expect these genes to experience relaxed selection or degeneration rather than positive selection, as has been observed in parasitic plants that lose photosynthesis genes. The loss of genes involved in gravitropism and lateral root development in these species is quite interesting.

Authors' response: We have also noticed this phenomenon, and our speculation is as follows: JKD is a putative nuclear-localized transcription factor belonging to the BIRDS/IDD C2H2-type zinc finger family. Together with its homolog BIB, JKD restricts SHR movement specifically to the endodermis, thereby defining the boundary of the root meristem by forming protein complexes. In plants, mutations in JKD lead to peripheral cell division in the cortex, increased numbers of cortical and epidermal peripheral cells, disruption of QC marker expression patterns, and disorder in QC and columella cell arrangements (Welch et al., 2007). SCR is expressed in the initial cortex/endodermis cells and endodermal cell lineages, regulating radial tissue organization in roots. SCR can form the SCR-SHR complex which activates the CYCD6 promoter, promoting its expression. CYCD6 is involved in asymmetric cell division of cortex/epidermal stem cells (Levesque et al., 2006). However, JKD and its homolog BIB inhibit the activity of the CYCD6 promoter activated by SCR-SHR (Long et al., 2006). JKD and SCR undergo positive selection in epiphytic bromeliads. It can be inferred that after positive selection, the resulting JKD, together with its homolog BIB, is sufficient to restrict the activity of the CYCD6 promoter activated by the SCR-SHR complex, reducing its expression below activated levels and thereby inhibiting asymmetric cell division of cortex/epidermal stem cells in epiphytic bromeliads roots. Additionally, JKD also acts on SHR, restricting its movement to the endodermis, ultimately leading to root degeneration in epiphytic bromeliads.

Welch D, Hassan H, Blilou I, Immink R, Heidstra R, Scheres B. Arabidopsis JACKDAW and MAGPIE zinc finger proteins delimit asymmetric cell division and stabilize tissue boundaries by restricting SHORT-ROOT action. Genes Dev. 2007;21(17):2196-2204. doi:10.1101/gad.440307

Levesque MP, Vernoux T, Busch W, et al. Whole-genome analysis of the SHORT-ROOT developmental pathway in Arabidopsis [published correction appears in PLoS Biol. 2006 Jul;4(7):e249]. PLoS Biol. 2006;4(5):e143. doi:10.1371/journal.pbio.0040143

Long Y, Smet W, Cruz-Ramírez A, et al. Arabidopsis BIRD Zinc Finger Proteins Jointly Stabilize Tissue Boundaries by Confining the Cell Fate Regulator SHORT-ROOT and Contributing to Fate Specification. Plant Cell. 2015;27(4):1185-1199. doi:10.1105/tpc.114.132407

6. A paragraph seems to be duplicated in the results, lines 399-416 and lines 417-436.

Authors' response: Thanks for bringing this to our attention. We made a mistake, and one of the paragraphs has been removed from the manuscript.

7. CYP96A15 P450s are involved in cuticular wax biosynthesis. The tandem duplication of this P450 in bromeliads is not unexpected, as P450s rapidly duplicate in plant genomes, but I do not see the link for how this gene is involved in trichome formation. Instead, I would assume this gene cluster is involved in cuticle formation on the already existing trichomes. Also, the in situ hybridization results in Figure 5c are not clear to me, and I cannot see trichome specific expression. For the spatial expression, is this a new experiment or data from the root data in Figure 4?

Authors' response: These genes are indeed involved in the biosynthesis of cuticular wax of trichomes. We have enlarged the results of the *in situ* hybridization images from Figure 5C to provide a clearer view of gene expression patterns. Additionally, the lack of cell type annotations may have caused confusion regarding trichome cells and the results. We have labeled the cell types in the section images. From the figures, it can be seen that these three genes are specifically expressed in the stock cells of epidermal hairs, with *CYP96A15-b* showing the highest expression level.

For the spatial expression, it is a new experiment from the data in Figure 6. The order of these images indeed was inappropriate; they should have been after Figure 6. We have moved these images to the SI as Supplementary Fig. 14.

Minor:

Line 57. It is generally not good to use the term lower plants. Similarly, what does 'advanced families' in line 65 refer to?

Authors' response: We corrected "lower plants" to "non-vascular plants", and corrected "advanced families" to "vascular plant families".

Line 194. What is genome completeness referring to here? BUSCO results, or assembly size compared to estimated genome size?

Authors' response: The genome completeness refers to the BUSCO results. We revised this sentence to avoid ambiguity.

Line 380. What does 'raw reads blasting' mean?

Authors' response: We revised this sentence from "The high-quality reference genome of *V. erythrodactylon* which was newly assembled in this study were subsequently employed for raw reads blasting" to "The high-quality genome of *V. erythrodactylon*

which was newly assembled in this study were employed as reference genome sequence".

Line 381. Is there a number missing for '111,73' or is the comma in the wrong place?

Authors' response: The comma is in the wrong place, which should be "11,173". We corrected it.

Line 338. I am not sure what 'SMART RNAseq is, and I could not find much via a google search. This should be defined here, as well as how it differs from typical RNAseq protocols/analyses.

Authors' response: Since it is challenging to collect a sufficient number of root samples from tillandsioid for traditional RNA-seq, we utilized a micro-transcriptome method known as Switching Mechanism at 5' end of RNA Template for RNA Sequencing (SMART RNA-seq). This method efficiently generates complete cDNA libraries from small sample amounts, enabling transcriptome analysis. We added the definition and detailed analysis in the SI lines 303-311.

Line 580. I am not a microbiome expert, but what does '1174-901-12' refer to, is this an un named genus?
Authors' response: No, *1174-901-12* is a genus name belonging to Rhizobiales-Beijerinckiaceae.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have made a good-faith effort in addressing many but not all of my concerns. I believe they can easily address the remaining concerns, which involve a number of important points.

Authors' response: Thank you so much for acknowledging our work. Please find our responses to your comments below.

1. OK, but core Tillandsioideae is never defined, and the citation of Barfuss et al. 2016 ignores the paper that coined the term. For clarity, grammar, accuracy, and assigning proper priority, I strongly recommend the following soft edits:

Lines 64-67, rewrite as: "… Unexpectedly, epiphytes are continuingly increasing with time, and are dominant today in a few large vascular plant families after recent massive expansions16-18.

Tillandsioideae, the largest subfamily of Bromeliaceae, includes roughly two-thirds of the epiphytic species within the family11,16,19,20." …

Notes: I recommend the change in the first sentence for proper usage. I recommend the change in the second sentence for accuracy and traceability; most English speakers would not interpret 65-66% as "nearly all", and Zotz 2016 (and 2013, from which I worked in writing this) provide the data for making a calculation of the numbers of epiphytic species in different bromeliad subfamilies.

Authors' response: Thanks for your suggestion. We have revised this sentence with proper usage as: "Unexpectedly, epiphytes have evolved to be dominant in a few large large vascular plant families after recent expansions" (Lines 64-65). And we have revised the second sentence for more accuracy as: "Tillandsioideae, the largest subfamily of Bromeliaceae, encompasses about two-thirds of the epiphytic species within the family" (Lines 66-67)

Lines 110-113, rewrite as: "Tillandsiodeae is often seen as consisting of the core tillandsioids20 and non-core tillandsioids, with the latter consisting of Catopsis and Glomeropitcairnia17,19. Our phylogenetic analysis did not sample Glomeropitcairnia but places Catopsis sister to the core Tillandsioideae …".

Note: I recommend the rewrite of the first sentence to give priority to Givnish et al. 2011 for coining the term "core tillandsioids", and for recognizing that group based on their multi-locus plastid phylogeny. Contrary to the authors' implication in their rebuttal, Barfuss et al. did not first recognize that group, but – as with Givnish et al. 2014 – used it subsequently, as have other later authors.

Authors' response: Thanks for your suggestion. We rewrote these sentences by giving priority to Givnish et.al (lines 112-115) as you suggested.

2. Good!

Authors' response: Thank you for acknowledging our work.

3. Good!

Authors' response: Thank you for acknowledging our work.

4. Perhaps. There is no ML reconstruction of character-states in Figure 1. I see either one gain of the atmospheric habit at the base of Tillandsia and five losses within Tillandsia, or two independent gains within Tillandsia and four losses. The first seems the more likely given the rarity of the atmospheric habit in angiosperms, but there is always the possibility of the same genetic background in Tillandsia resulting in independent acquisitions of the trait – only detailed analyses of the genes involved would give a definite answer. The authors should state those possibilities.

But the caption to Figure 1 MUST be clarified. Line 1102 is inadequate. What is a "living habitat"? What are states m, sx, and x within that character? What are the sources of the data? Give a key in the legend – horizontally, about approximately 250° – with labels Species, Habitat, and Photosynthetic pathway. Below each of those headings (which should align with the circles around the tree), give colored boxes and keys, and then get rid of CAM, C3 (note that the subscript MUST be used throughout the manuscript!), m, xs, and x in the circles. Color coding is enough, and IMO nobody looking at a print version of the figure will be able to read those tiny labels.

Authors' response: Thanks for your suggestion. We added a statement about the origin of atmospheric habit in the manuscript lines 129-132, and modified the Fig.1 as you suggested.

We are really sorry for the confusion. We should use "habit" rather than "habitat." Habits are classified according to the descriptions by Gilmartin (1983) and Barfuss et al. (2012). The classifications are as follows: M (Mesic or Mesomorphic): Plants with ligulate leaves featuring few, inconspicuous, appressed scales or trichomes, have a tank without water-storing tissue; SX (Semixeric or Semi-xeromorphic): Plants with poorly developed tanks or no tanks, narrow leaf blades with inconspicuous, appressed scales, and little or no water-storing tissue; X (Xeric or Xeromorphic): Plants with conspicuous, spreading trichomes, narrow leaf blades, water-storing tissue, and no tank.

These data were obtained by observation according to classification criteria, and most of them were also verified by literature review. We added the clarification criteria in SI methods lines 24-26 and the Fig. 1 legends lines 1118-1223.

5. It is good that the authors have largely eliminated their use of "transition" or "transitional" when talking about organs, given that – so far as I can see – only gains and losses are discussed in this paper. But they leave two statements that are glaringly inconsistent with this perspective in prominent places, at lines 75-76 in the Introduction and at lines 594-596 at the very beginning of the Discussion (lines 594-596). There is no justification, so far as I can see, for these statements and I must insist they be removed or rephrased. There is no documentation of "transitional forms", and there is no demonstration of the "gradual loss of old ones" following the acquisition of new organs. And what are the "new organs" to which the authors refer?

Authors' response: Thanks for your valuable comments. We have removed these sentences from the manuscript accordingly.

We are sorry for the inaccurate description. “new organs” is inappropriate. What we want to say is “new function of organs”. Anyway, we removed this word from the manuscript.

6. Good.

Authors’ response: Thank you for acknowledging our work.

7. “Air plant” is now used 40 times in the text. Don’t change one instance and then claim you’ve met a reviewer’s concern!

Authors’ response: We are sorry for our ignorance. Actually, atmospheric *Tillandsia* are often known as “air plants.” However, we have updated the manuscript to use “epiphytes”, “atmospheric epiphytes” or “tillandsioids”, while only retaining “air plant” in line 76 and line 1238 to make it easier for the reader to understand by vivid description.

8. OK in general, but I do not understand what the authors mean on lines 609-610 by “from the perspective of clades or taxonomic groups”. Perhaps the sentence could be rewritten as “But in terms of broader clades, our nuclear phylogeny is consistent with the (mostly) plastid phylogeny of Barfuss et al.” Is that accurate? Is the pattern of relationships among those component clades comparable to that seen in the Barfuss phylogeny? If not, please write an informative and accurate description instead.

Authors’ response: Thanks for your suggestion. We rewrote this sentence as you suggested in lines 623-625.

9. Good!

Authors’ response: Thank you for acknowledging our work.

10. The authors must rewrite lines 643-646 (and any associated text in the narrative or SI) to make it clear whether increased or decreased thermal seasonality favors tank evolution, and whether increased or decreased temperature in the coldest quarter favors atmospheric evolution. Adding a couple of words would make all the difference between a highly informative statement and a muddle.

Authors’ response: Thanks for your comments. In fact, we cannot determine whether an increase or decrease in these factors favors the evolution of these plants. Because according to the description of MaxEnt models, temperature seasonality (Bio_4) refers to changes in thermal seasonality, meaning that both increased and decreased thermal seasonality may influence tank plant evolution. Similarly, a dramatic change (whether an increase or decrease) in temperature during the coldest quarter may have driven the evolution of atmospheric plants. We rewrote the descriptions in the manuscript lines 155-157 and lines 655-660 to make it more clear.

11. The authors cited the wrong reference! The correct one is Givnish, T. J., K. J. Sytsma, J. F. Smith, W. J. Hahn, D. H. Benzing, and E. M. Burkhardt. 1997. Molecular evolution and adaptive radiation in Brocchinia (Bromeliaceae: Pitcairnioideae) atop tepuis of the Guayana Shield. Pp. 259-311 in T. J. Givnish and K. J. Sytsma (eds.), Molecular Evolution and Adaptive Radiation. Cambridge University Press, New York.

Authors’ response: Sorry for the wrong citation. We corrected it.

12. Good!

Authors' response: Thank you for acknowledging our work.

13. Disagree. If there is a waxy cuticle over the trichome cap cells – which is what you describe – it is not clear how water could enter them and, then, enter the live stem cells and supply moisture to the leaves. Contrary to what the authors wrote, Raux et al. do NOT show a waxy cuticle over the trichome cap cells – see their figure 1e and 1f.

Authors' response: Thanks for your questioning. We checked the reference again by Raux et al. Although Raux et al. do not show a waxy cuticle on the trichome cap cell, they do show cuticle on the dome and foot cells (fig. 3d). They found "Thin cuticle layer lining the lateral walls of the dome cell and trichome stalk." Our *in situ* observations also indicated that these three genes are expressed in the dome and foot cells, not in the wing or cap cells (Fig. 5c). In addition, according to their finding, "The *Tillandsia* trichomes achieve this with a precisely laid down cuticle covering the lateral walls of the dome cell and foot cells." They suggest that the cuticle covering the lateral walls of the dome cell and foot cells could prevent capillary flow within the wall space connecting the outer trichome to the mesophyll. According their theory, the cuticle of trichome dome cell and foot cells is crucial for forming the trichome structure needed for its absorption function. However, these cuticle function for bromeliads needs further detailed study in the future. Anyway, thanks for raise this good question for discussion. This led us to think more deeply about the role of the cuticle in bromeliad plants. We added several sentences in the manuscript lines 443-447 and lines 693-695 to avoid any confusion.

14. Why is Pierce et al. 2021 cited? Why isn't Givnish et al. 1997 named in the text for this pioneering discovery?

Authors' response: Sorry for the wrong citation. What we want to cite is Brighigna et al., 1992, not Pierce et al. 2021. We corrected this citation and rewrote this sentence in the manuscript line536.

Brighigna, L., Montaini, P., Favilli, F., & Trejo, A. C. (1992). Role of the nitrogen-fixing bacterial microflora in the epiphytism of Tillandsia (Bromeliaceae). Am J Bot, 79(7), 723-727.

15. Good!

Authors' response: Thank you for acknowledging our work.

16. The authors did NOT cite Givnish et al. 2014 in the introduction as recommended, on a crucial point where it might appear that they are themselves reaching that conclusion. I strongly suggest they do so now, inserting this sentence at line 74: "Givnish et al. (2014) showed that tank formation evolved first in Tillandsioideae, with atmospheric species later evolving in Tillandsia."

Authors' response: Thanks for your valuable recommendation. We inserted this sentence in the manuscript lines77-79.

I will defer to the other reviewers to address the authors' responses to their suggestions.

Respectfully submitted,

Thomas J. Givnish

Henry Allan Gleason Professor of Botany and Environmental Studies

Wilhelm Hofmeister Professor of Botany

University of Wisconsin-Madison

Reviewer #2 (Remarks to the Author):

I thank the authors for their work in revising the manuscript. Overall, most of my concerns have been sufficiently addressed. A few small points remain:

Authors' response: Thank you for acknowledging our work. Please find our responses to your comments below.

1: Thanks for the clarification about "phyllospheric" bacteria, and I understand. It would be useful to make this very clear given phyllospheric here means for atmosphere types. Adding a sentence making this point would help as many plant microbiome researchers will assume you are differentiating between root and leaf tissues.

Authors' response: Thanks for your valuable suggestion. We added the clarification about "atmospheric plants" and "phyllospheric bacteria" in lines 75-77 and line 541 to avoid any confusion.

2. Thanks for redoing the analysis with the plastids removed and adding more detail to the methods for the microbiome analyses. I apologize that I was unclear about my comment regarding rarefaction and accounting for read depth. PCoA and PERMANOVA analyses can be sensitive to the overall read number per sample. There are pros and cons to rarefying data, but if you don't rarefy, then you should include read counts as a covariate in your PERMANOVA analysis. While LEfSe uses relative abundance, it would also help just to show that microbiomes were sampled at sufficient read depth. To do this, providing rarefaction curves as supplementary figure would help.

Authors' response: Thanks for your suggestion, and we have provided rarefaction curves in Supplementary Fig. 15a.

Reviewer #3 (Remarks to the Author):

In this revision, the authors provided additional detail on their methods, re-analyzed their microbiome data at the ASV level to the same qualitative conclusion, removed host plastid sequences, and compared the taxonomic composition of their tillandsioid microbiomes to an outgroup. The revised version of the microbiome analysis is now much stronger, and still supports their claim that nitrogen-fixing bacteria may play a particular role in plant nutrition for these species. I have only a few minor comments about remaining typos:

Authors' response: Thank you for acknowledging our work. Please find our responses to your

comments below.

Line 50: change ‘have’ to ‘has’

Authors’ response: We changed ‘have’ to ‘has’.

Line 64: change ‘increasing’ to ‘expanding’

Authors’ response: Thanks for your suggestion. We have rewritten this sentence according to the comment of Reviewer 1.

Line 99: change ‘helpful’ to ‘help’ or add ‘be’

Authors’ response: We changed ‘helpful’ to ‘help’.

Line 510: I don't understand the usage of the word "asymbiotic" here. Is this in reference to bacteria that do not reside in intracellular compartments? I can't tell from the methods how bacterial DNA was isolated from plants, but if there was a tissue homogenization step (as opposed to washing cells off the surface) then the bacterial sequences could very well be derived from intracellular symbionts. In any case, this seems like an unnecessary specification that could be removed from the section title.

Authors’ response: Thanks for your suggestion. We removed the inappropriate word "asymbiotic" from the manuscript.

SI Line 387: change 'arter' to 'after'

Authors’ response: We changed 'arter' to 'after'.

Reviewer #4 (Remarks to the Author):

the authors have addressed by previous concerns and I appreciate their detailed revisions.

Authors’ response: Thank you very much for acknowledging our work.

Reviewer #1 (Remarks to the Author):

The authors have satisfactorily addressed all of my concerns. My thanks to them!

I do think they should determine, by inspecting maps of thermal seasonality and of the distribution of tank bromeliads (or atmospheric bromeliads), whether those kinds of epiphytes increase with seasonality, decrease with seasonality, or are dominant at an intermediate level of seasonality. Just tweaking a couple of words, based on such observations, would strengthen their ecological contribution.

Authors' response: Thank you so much for acknowledging our work and your valuable suggestion. According to your suggestion, we inspected the maps of distribution of two types of bromeliads in Middle Pliocene and Late Pleistocene (Fig. 2b). Compared to the Middle Pliocene, when global seasonal temperature differences were lower, the distribution of tank bromeliads increased in the Amazon and Central America but decreased in the Brazilian Shield during the Late Pleistocene, a period characterized by higher seasonal temperature differences. The uplift of the Brazilian Shield during this time may have reduced local seasonality. By accounting for this variable, we speculate that tank bromeliads thrive in regions with greater seasonality. In contrast, atmospheric bromeliads increased in Central America, the Amazon, and the Brazilian Shield, with only a slight decrease observed in a small part of the Andes during the Late Pleistocene, which experienced lower temperatures in the coldest quarter compared to the Middle Pliocene. We therefore hypothesize that the increase in atmospheric bromeliads is associated with the decrease in temperature during the coldest quarter.

According to your suggestion, we tweaked some words in the discussion lines 652-662

Reviewer #2 (Remarks to the Author):

I thank the authors for their work in the revision in this fascinating system. My concerns have been sufficiently addressed, nothing else to add.
Authors' response: Thank you so much for acknowledging our work.

Reviewer #3 (Remarks to the Author):

I support publication of this manuscript.
Authors' response: Thank you so much for acknowledging our work.

Lyu et al. present a remarkably integrative analysis of evolution in the large bromeliad subfamily Tillandsioideae, including phylogeny, historical biogeography, climate niche evolution, genomic bases of key functional traits, and identification of N-fixing microbes associated with several epiphytic species. This paper makes several important contributions to our understanding of bromeliad evolution, and opens up new avenues of inquiry that are likely to be highly productive in the future.

However, in my opinion, a few of the authors' key conclusions are unwarranted, and the authors' fail to acknowledge some previous conclusions that parallel their own. These errors must be corrected.

Here are my detailed comments:

1. The Abstract states that the tillandsioids arose in the Andes. This claim is based on analyses and statements on lines 137-139. In my opinion, this conclusion is not justified and is an artifact of inadequate sampling of ingroups and outgroups. Givnish et al. 2011 – not cited re historical biogeography in either the text or SI, and which included representatives of **all** bromeliad subfamilies – concluded that the **core Tillandsioideae** (which excludes the *Catopsis-Glomeropitcairnia* clade) arose in the Andes. It is obvious that the conclusion by Lyu et al. is an artifact of failing to include representatives of three key outgroups (bromeliad subfamilies Brocchinioideae, Lindmanioideae, and Navioideae) as well as the non-Andean ingroup *Glomeropit-cairnia*. The two species of *Catopsis* they did include are restricted to Central America. A representative sampling of bromeliad ingroups and outgroups thus would not support an Andean origin for the tillandsioids. Consequently, the authors' conclusions re the region where Tillandsioideae arose are untenable. Furthermore, they fail to cite the biogeographic conclusions of Givnish et al. 2011, which bear directly on the question they attempted to address. If they correct their biogeographic analysis, they should also cite that paper as having reached the same conclusion 15 years ago.

2. In the SI, the authors state that four fossil dates and a secondary calibration point were used to date their phylogeny (bottom, first page). However, none of these dates are identified. Those dates must be stated explicitly!

3. Line 62: "Remarkably, epiphytes have evolved among all **major lineages** of land plants" – this change is necessary given the incorrect conclusions that could be drawn from the vague "all taxa of land plants" used by the authors.

4. Line 148: "Since the emergence of atmospheric bromeliads…" – Given the authors' sampling within Tillandsioideae, it does appear that there was a single origin of the atmospheric habit within *Tillandsia* (the only genus with atmospheric species) with only a few reversions to the tank habit. I am not an expert on *Tillandsia* diversity, but I found this conclusion surprising given what I know about the distribution of atmospherics in the Barfuss et al. plastid phylogeny. I do hope that Michael Barfuss

has been consulted on this issue. If there is a single origin of the atmospheric habit … which is not unreasonable from an evolutionary viewpoint … that would be a big contribution of this paper. So, please confirm this point.

5. The discussion of tillandsioids growth forms (lines 67-85) needs to be tightened up. First, Tillandsioideae is not merely "a prominent epiphytic subfamily", it is the largest and almost all its species are epiphytic. The only other subfamily with substantial numbers of epiphytics is Bromelioideae. Second, the phrase about tillandsioids exceeding even Orchidaceae "in range" should be rewritten so as to avoid erroneous impressions by the readers – orchids have a far greater geographic distribution than bromeliads as a whole. Third, both atmospheric and tank epiphytes in Tillandsioideae have absorptive trichomes (see Benzing's books and publications re this topic. The authors' wording implies this may not be true. Fourth, lines 76-79 require phylogenetic reconstructions of trait evolution that have never been conducted; this is plausible speculation, but speculation nonetheless. Finally, lines 79-85 imply that we're going to see analyses of transitional forms, possibly including the transition to epiphytism, given how prominently that habit features in the discussion thus far. However, the tillandsioids do not – to my knowledge – provide any system for analyzing the transition to epiphytism, given that epiphytism is ancestral to Tillandsioideae, as shown by Givnish et al. 2014 (not cited in ms.).

6. Line 86: get rid of "tachylalic" and insert "rapid".

7. Line 89: get rid of "air plant" and use either of the two well-defined terms "epiphyte" or "atmospheric epiphyte" so that the readers know what is being talked about.

8. Line 95: One of the most important aspects of this paper is unannounced in the abstract AND here – namely, that the new phylogeny presented is based on (largely) nuclear transcriptomic sequences. Please remedy this! This novel basis, however, requires some statements in the Results and prep in the SI methods re what broad-scale differences the authors see between their nuclear phylogeny and earlier, even more thoroughly sampled phylogenies based on plastid genes by Barfuss and his colleagues.

9. Lines 104-110: Apparently, based on the elliptical statements in the SI, the authors applied maximum likelihood on the concatenated data to obtain their nuclear phylogeny. Fine. BUT there are several shortcomings: (a) I do not see bootstrap support values anywhere; that must be remedied, perhaps in the SI. As it is, we cannot assess the extent to which the authors' findings are definitive. (b) It is standard procedure to present (and, often, to use) ASTRAL analyses of nuclear data, to move from gene trees to species trees – this should include some analyses of discordance. (c) Some statement MUST be made about how the Lyu et al. nuclear phylogeny differs from that advanced by Barfuss et al. 2012 and 2016.

10. Lines 160-162: Specify the actual nature of the association of each growth form with each environmental variable. For example, is the atmospheric habit associated with colder or warmer temperatures?

11. Lines 309-311: The authors should cite Givnish et al. 1997 (in Givnish & Sytsma, Molecular Evolution and Adaptive Radiation), who over 25 years ago identified the connection of the tank habit to soft, easily conformable leaves and broad leaves (e.g., "the tank habit, perhaps because the latter requires relatively soft, broad leaves that conform tightly to each other …").

12. Lines 321-323: The authors should cite Benzing 2000, at least, to support this statement.

13. Lines 459-461: The apparent claim that all trichomes have a waxy cuticle is supported, as far as I can see, by studies outside Bromeliaceae. Furthermore, the death of trichomes exposed to desiccation in *Brocchinia* (Givnish et al. 1997) argues against this as a universal rule. Perhaps it is my ignorance, but I have seen studies of trichomes in tank epiphytes in Tillandsioideae and Bromelioideae to know if their live trichomes are covered with wax. I raise this question because the presence of a waxy cuticle might interfere with water or nutrient uptake by trichomes.

14. Lines 536 and following: This section is, to me, one of the most interesting contributions in the paper. However, the authors should cite Givnish et al. 1997 for the presence of N-fixing cyanobacterial in the tanks of certain *Brocchinia* species.

15. Line 626: Again, origin of tillandsioids in the Andes is NOT supported!

16. Discussion: Here, and in the intro, the authors should cite Givnish et al. 2014, who found that epiphytism in Bromeliaceae always arose in tank-forming species. Here, and in the intro, the authors should also cite Givnish et al. 2014 and Givnish et al. 2015 (Proc R Soc B) for the general finding that epiphytism accelerates species diversification, as well as their interpretation of why that occurs.

I waive my right to anonymity as a reviewer.

Sincerely yours,

Thomas J. Givnish
Henry Allan Gleason Professor of Botany
Wilhelm Hofmeister Professor of Botany
University of Wisconsin-Madison

Review of Lyu et al., v2

The authors have made a good-faith effort in addressing many but not all of my concerns. I believe they can easily address the remaining concerns, which involve a number of important points.

1. OK, but core Tillandsioideae is never defined, and the citation of Barfuss et al. 2016 ignores the paper that coined the term. For clarity, grammar, accuracy, and assigning proper priority, I strongly recommend the following soft edits:

   Lines 64-67, rewrite as: "… Unexpectedly, epiphytes are continuingly increasing with time, and are dominant today in a few large vascular plant families after recent massive expansions[16-18].
   Tillandsioideae, the largest subfamily of Bromeliaceae, includes roughly two-thirds of the epiphytic species within the family[11,16,19,20]." …

   Notes: I recommend the change in the first sentence for proper usage. I recommend the change in the second sentence for accuracy and traceability; most English speakers would not interpret 65-66% as "nearly all", and Zotz 2016 (and 2013, from which I worked in writing this) provide the data for making a calculation of the numbers of epiphytic species in different bromeliad subfamilies.

   Lines 110-113, rewrite as: "Tillandsiodeae is often seen as consisting of the core tillandsioids[20] and non-core tillandsioids, with the latter consisting of *Catopsis* and *Glomeropitcairnia*[17,19]. Our phylogenetic analysis did not sample *Glomeropitcairnia* but places *Catopsis* sister to the core Tillandsioideae …".

   Note: I recommend the rewrite of the first sentence to give priority to Givnish et al. 2011 for coining the term "core tillandsioids", and for recognizing that group based on their multi-locus plastid phylogeny. Contrary to the authors' implication in their rebuttal, Barfuss et al. did not first recognize that group, but – as with Givnish et al. 2014 – used it subsequently, as have other later authors.

2. Good!

3. Good!

4. Perhaps. There is no ML reconstruction of character-states in Figure 1. I see either one gain of the atmospheric habit at the base of *Tillandsia* and five losses within Tillandsia, or two independent gains within *Tillandsia* and four losses. The first seems the more likely given the rarity of the atmospheric habit in angiosperms, but there is always the possibility of the same genetic background in Tillandsia resulting in independent acquisitions of the trait – only detailed analyses of the genes involved would give a definite answer. The authors should state those possibilities.

But the caption to Figure 1 **MUST** be clarified. Line 1102 is inadequate. What is a "living habitat"? What are states m, sx, and x within that character? What are the sources of the data? Give a key in the legend – horizontally, about approximately 250° – with labels Species, Habitat, and Photosynthetic pathway. Below each of those headings (which should align with the circles around the tree), give colored boxes and keys, and then get rid of CAM, C_3 (note that the subscript MUST be used throughout the manuscript!), m, xs, and x in the circles. Color coding is enough, and IMO nobody looking at a print version of the figure will be able to read those tiny labels.

5. It is good that the authors have largely eliminated their use of "transition" or "transitional" when talking about organs, given that – so far as I can see – only gains and losses are discussed in this paper. But they leave two statements that are glaringly inconsistent with this perspective in prominent places, at lines 75-76 in the Introduction and at lines 594-596 at the very beginning of the Discussion (lines 594-596). There is no justification, so far as I can see, for these statements and I must insist they be removed or rephrased. There is no documentation of "transitional forms", and there is no demonstration of the "gradual loss of old ones" following the acquisition of new organs. And what are the "new organs" to which the authors refer?

6. Good.

7. "Air plant" is now used 40 times in the text. Don't change one instance and then claim you've met a reviewer's concern!

8. OK in general, but I do not understand what the authors mean on lines 609-610 by "from the perspective of clades or taxonomic groups". Perhaps the sentence could be rewritten as "But in terms of broader clades, our nuclear phylogeny is consistent with the (mostly) plastid phylogeny of Barfuss et al." Is that accurate? Is the pattern of relationships among those component clades comparable to that seen in the Barfuss phylogeny? If not, please write an informative and accurate description instead.

9. Good!

10. The authors **must** rewrite lines 643-646 (and any associated text in the narrative or SI) to make it clear whether increased or decreased thermal seasonality favors tank evolution, and whether increased or decreased temperature in the coldest quarter favors atmospheric evolution. Adding a couple of words would make all the difference between a highly informative statement and a muddle.

11. The authors cited the wrong reference! The correct one is Givnish, T. J., K. J. Sytsma, J. F. Smith, W. J. Hahn, D. H. Benzing, and E. M. Burkhardt. 1997. Molecular evolution and adaptive radiation in *Brocchinia* (Bromeliaceae: Pitcairnioideae) atop tepuis of the Guayana Shield. Pp. 259-311 in T. J. Givnish

and K. J. Sytsma (eds.), *Molecular Evolution and Adaptive Radiation*. Cambridge University Press, New York.

12. Good!

13. Disagree. If there is a waxy cuticle over the trichome cap cells – which is what you describe – it is not clear how water could enter them and, then, enter the live stem cells and supply moisture to the leaves. Contrary to what the authors wrote, Raux et al. do NOT show a waxy cuticle over the trichome cap cells – see their figure 1e and 1f.

14. Why is Pierce et al. 2021 cited? Why isn't Givnish et al. 1997 named in the text for this pioneering discovery?

15. Good!

16. The authors did NOT cite Givnish et al. 2014 in the introduction as recommended, on a crucial point where it might appear that they are themselves reaching that conclusion. I strongly suggest they do so now, inserting this sentence at line 74: "Givnish et al. (2014) showed that tank formation evolved first in Tillandsioideae, with atmospheric species later evolving in *Tillandsia*."

I will defer to the other reviewers to address the authors' responses to their suggestions.

Respectfully submitted,

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