The authors provide an exceptionally sampled dataset giving us a fine-scale resolution of the distribution of black-bellied and shovel-nosed *Desmognathus* in and around Great Smoky Mountains National Park. It is phenomenal to see a resource like this, and I can only hope the authors (and other researchers) will continue such surveys in the future.

However, I have four major concerns regarding how the data are presented, how the phylogenetic dating was performed, how species boundaries are discussed, and whether the sequences for the new "Cades Cove" lineage are valid. The sequences included in the supplementary file do not appear to conform to the biological expectation of open reading frames and proper triplet counts for indels, nor do their patterns of nucleotide substitution match our expectation for mitochondrial dynamics in salamanders. I suspect that they might be nuclear-mitochondrial insertions from an ancient hybridization event between the Nantahala and Pisgah clades. It is imperative that the authors address these concerns of biological validity, although even if the sequences are nuMts, they would still likely be valid to show the patterns of geographic co-occurrence that are the primary focus of the MS.

One small note: it was very easy to overlook in their supplemental materials, but Pyron et al. 2025 did show that *D. gvnigeusgwotli* and *D. mavrokoilus* are in the same stream system near the Pigeon River outside of the NW margin of GRSM. RAP1686-9/AMNH A-195035-8 are *D. gvnigeusgwotli* from Stinking Camp Branch (35.731729 -83.058740), while RAP1690-1/AMNH A-195329-30 are *D. mavrokoilius* from Dogwood Flats Creek tributary (35.711306 -83.068138), all draining into the same stream leading into the Pigeon River.

1. Change the clade nomenclature

My first comment is to *implore* the authors to jettison or reduce the use of the Jackson 2005 A/B/C nomenclature, which will introduce substantial confusion into the literature and dramatically obscures and undermines the importance of the authors' conclusions. I understand why they did this initially – to be internally logically consistent with the topological position of samples recovered from mitochondrial data as described previously by Jackson 2005 – but it creates monumental confusion when trying to cross-reference the valid species names, their phenotypes, their phylogenetic position, and their distributions.

For background, Jackson 2005 – in an unpublished MS thesis – introduced an informal A/B/C clade designation to refer to the unusual patterns of phylogenetic relatedness in black-bellied and shovel-nosed salamanders. This never became widely used.

Also in 2005, Kozak et al. 2005 introduced a widely used nomenclature using species names and A/B/C, such that *D. amphileucus* was – at the time – "quad/marm A," while *D. aureatus* was "quad/marm B," *D. mavrokoilius* and *D. intermedius* were "quad/marm C," etc. This was expanded by Beamer and Lamb 2020 and has been used in dozens of publications, perhaps most prominently Pyron et al. 2020/2022, who demonstrated the species-level distinctiveness of these lineages.

Similarly, Jones and Weisrock 2018 introduced the valid and more euphonious and memorable “Nantahala” and “Pisgah” clade names for the divergent nuclear position of the northern and southern clades in the complex, which have also become widely used.

For the present authors to reintroduce the Jackson 2005 A/B/C nomenclature is a regression of more than 20 years of painstaking work to clarify the phylogenetic relationships and taxonomic status of these populations, as well as undermining the authors’ own stated goals from their abstract – “to clarify distributions and patterns of co-occurrence of recently described species in the Great Smoky Mountains.”

It seems imperative that the authors rely on the valid species names first and foremost in all descriptions of their results, to underscore the nature of the patterns they recovered. Use of “Nantahala” and “Pisgah” also seems desirable to clarify the distinct phylogenetic positions of these groups, which are obscured by the mitochondrial data due to known discrepancies revealed by phylogenomic analysis.

This can be seen most clearly in the figures such as figure 2 and 3. The illustration of ranges in figure 2 is very misleading since it lumps multiple valid, described species together under clade names that can’t be connected to any of the recent phylogenetic, nomenclatural, or taxonomic work on these groups, while also failing to convey the relevant phenotypic information. For instance, “B & C together” dots really mean *D. amphileucus, D. aureatus* and *D. folkertsi,* which are a shovel-nosed and 2 black-bellied, while “Clade B” dots are two black-bellied species, *D. amphileucus* and *D. gvnigeuswotli*.

But those shovel-nosed Clade C dots are “quad/marm B,” while the Clade B dots are “quad/marm A” and “F,” and all are in the Nantahala Clade, while Clade A contains “quad/marm” C/D/E, otherwise known as *D. mavrokoilius, D. kanawha, D. marmoratus,* and *D. intermedius* in the Pisgah Clade, both black-bellied and shovel-nosed. Hopefully it’s clear why this is an untenable nomenclatural system, as almost nobody could keep this straight. In fact, I’ve had an extensive discussion with Paul Super, the science coordinator at GRSM, having to explain at length why Jackson 2005 “marmoratus” in “Clade A” were actually “marm C” (i.e., *D. intermedius*) and not “quad A” (*i.e., D. amphileucus*) and that Clade C “marmoratus” were actually “marm B” (*D. aureatus*) in the Nantahala clade, not “marm C” *D. intermedius*! Please just use the species names.

This comes back in force for figure 3, where a reader must attempt to hold in their minds what BB/SN and Clade A/B/C mean when interpreting the maps. Please spell out black-bellied and shovel-nosed, use the species names (*D. gvnigeusgwotli* and *D. mavrokoilius* for black-bellied and *D. intermedius* and "unknown lineage" for shovel-nosed), and provide a legend key that breaks them up by Nantahala and Pisgah. The colors in figure 4 also don't match those in figures 2 and 3. It is burying the authors' lede to not have figure 3 clearly drive home that "we found extensive co-occurrence of the black-bellied *D. gvnigeusgwotli* (Nantahala) and *D. mavrokoilius* (Pisgah) across the NW side of the Park – two distantly related but morphologically similar species which can apparently exist in sympatry."

Even in the authors' text it's unclear, as they say things like "Both Jackson (2005) and Jones and Weisrock (2018) detected introgression between BB and SN ecomorphs in clade A but no evidence of introgression between ecomorphs in clades B and C (Nantahala), and it seems clear that there is no gene flow between northern (clade A) and southern (clades B and C) lineages (Pyron et al., 2020)." This sentence shifts between calling it the Nantahala, "Southern," or "B and C" clades, when Pyron et al. 2020 called it Nantahala, and not mentioning that Clade A is Pisgah. Please call them Nantahala and Pisgah for clarity.

2. Abandon or modify the dated analysis

The dating analysis presented is not sufficiently robust to support the level of confidence expressed by the authors and should be minimized or removed. First, 375bp of a single mitochondrial gene does not contain sufficient signal to parameterize a robust molecular-clock analysis. Second, the authors overlook the results of Budd and Mann (2024; Syst. Biol.) and Pennell (2023; Nature) regarding the insufficiency of these types of analyses for estimating species' origins. Third and most importantly, the authors are violating the assumptions of the models by including population-level sampling across species. The prior densities and parametric models used in relaxed clock dating generally assume that tips are species, such that the distribution of substitutions along branches can be modeled as a Poisson process with a rate parameter describing inter-specific variation.

Including multiple individuals within species violates this since the expectation of variable sites occurs with a much lower rate, inducing a mixture process in the posterior that isn't accounted for in the prior. Consequently, the branch lengths, clock rates, and divergence times are distorted in artifactual ways that renders them imprecise and inaccurate. This will be compounded immensely by the tiny amount of sequence data present. I realize that this strategy is very common, and the authors are only following many recent papers, but they are all generally suspect, and this strategy is generally untenable for dating divergences at the scale the authors propose here. Consequently, most of the "species ages" estimated by the authors are simply sitting mid-way from the root to the tip, as the posterior can't meaningfully diverge from the prior. One thing the authors could do is sample the prior to demonstrate a shift, but if present, this would still not satisfy the issue of the prior assumptions being violated. I think the paper still stands fine without such an analysis.

3. Be more circumspect about species boundaries

I also take issue with how the authors present discussion of species boundaries. They are laudably conservative in many instances, but I think they give the impression of too much interpretation of their limited mitochondrial data, and an incomplete synthesis of the existing literature. As they note, all nuclear and mitochondrial data support essentially total isolation and firm species boundaries between the Nantahala *D. amphileucus, D. aureatus, D. folkertsi,* and *D. gvnigeusgwotli,* with no apparent gene flow between any of those species or with any lineages in the Pisgah clade.

Within the Pisgah clade, *D. intermedius* exchanges genes and mitochondria with geographically adjacent popuations of *D. mavrokoilius* C (but not E or G), and *D. marmoratus* (not addressed in this study) likewise does so with *D. mavrokoilius* E and G, but not C. This is addressed in detail in Pyron et al. 2022, 2025. Finally, *D. kanawha* exhibits only limited past apparent gene flow with *D. mavrokoilius,* but not the shovel-nosed species. It is not accurate to say “Subsequently, Pyron et al. (2022) supported this interpretation with a larger dataset, but also presented evidence of mixed ancestry across all groups within clade A, consistent with extensive hybridization between ecomorphs.” We did not present evidence of mixed ancestry between *all* groups, but only a limited subset of geographically adjacent populations.

Similarly, the authors state “Jones and Weisrock (2018) suggested that BB and SN ecomorphs in clade A could not be considered two distinct species, instead proposing genetic polymorphism or developmental differences as potential explanations for the two ecomorphs." However, they do not mention the extensive testing and compelling findings of Pyron et al. 2020 who showed that Jones and Weisrock 2018 did not actually sample any shovel-nosed individuals in their dataset and misidentified many specimens! The unpublished PhD thesis of Jones 2023 acknowledges this: https://uknowledge.uky.edu/biology_etds/92/

The authors then state that “This is consistent with random mating between clade A phenotypes within streams.” This is not true. Random mating within streams would produce small numbers of identical haplotypes shared between morphotypes given their fast coalescence, but there is substantial variation in haplotype diversity that stretches across streams and between morphotypes. Furthermore, Pyron et al. 2022 conclusively demonstrated that most populations include individuals of “pure” ancestry (>80%) for each morphologically congruent lineage, which would be impossible if they were in *pan mixia* in individual streams. Individuals with hybrid ancestry are a minority.

I realize the authors are trying to hedge when they say “However, any single locus might be misleading, and our sample sizes are too modest to detect correlations between ancestry and phenotype much weaker than about 0.64 (Cohen, 1988). Thus, our data do not clearly differentiate whether clade A ecomorphs should or should not be classified as separate species but do highlight the need for continued work in this system.” But I think this entire section is unnecessary and could be removed – the authors data simply don’t permit speculation on species boundaries, especially given what is known about the extensive genealogical cohesion of these species from more extensive phylogenomic datasets.

The authors also state several times that their data reveal a high *rate* of hybridization, but this is not correct – these data cannot inform us as to the frequency with which hybridization takes place. What they reveal is that there is a high proportion of shared mitochondrial variation between the morphotypes. But these could have derived from a very small number of ancestral hybridization events and subsequent spread of captured mitochondria across populations with differential drift. This is already known from *D. lycos* and *D. carolinensis* (Beamer and Lamb 2020; Pyron et al. 2020, 2022).

Finally, while it is true that we said “These two BB species cannot be distinguished by any known visual characteristics (Pyron & Beamer, 2022),” this is not really accurate as presented – we meant diagnostically with certainty. It is quite easy to differentiate *D. gvnigeusgwotli* and *D. mavrokoilius,* even in the field. We provided Table 4 giving a range of qualitative color-pattern characteristics that can serve to differentiate them.

4. Further investigate the unusual sequences

The authors purport to discover a new Nantahala lineage of shovel-nosed in Cades Cove, going so far as to call it “sp. nov.” in Table 1. This is dramatically premature, as they offer no specimens, photos, measurements, vouchers, descriptions, etc. Using “sp. nov.” is usually paired with a description, but this is not given. More data would be needed before concluding that this is a distinct lineage, let alone a new species. In their tree, they don’t even show a “lineage” – implying monophyly – but a polytomy with multiple samples. Nuclear genomic data will be needed to clarify this, in comparison with existing datsets. They also don’t clearly explain why the Cades Cove shovel-nosed populations are a new lineage/species, but not the distinct clade of Smokies black-bellied populations that has approximately the same amount of divergence. However, this is not the main issue.

Unless I am making some mistake, the mitochondrial sequences presented for these specimens don't appear to be valid cytochrome *b* sequences from a *Desmognathus*. I base this conclusion on several points.

First, the sequences as presented are not in open reading frame from any codon position – any translation origin from 1 to 3 reveals internal stop codons.

Second, when aligned to other *Desmognathus,* there is a 10bp internal deletion induced, present in all other *Desmognathus,* which brings the sequences into "reading frame" before and after the indel but obviously isn't biologically plausible since it's not divisible by 3. After aligning with outgroups (see below), this indel is absent from all batrachians. Finally, the 10bp frameshift/indel is found in all 255 of the GRSM sequences in the file provided by the authors, but they do not remark on this in the MS to provide an explanation.

Third, while the specimens come out as "sister" to *D. aureatus* + *D. folkertsi* in the phylogenetic analysis they only appear to share 3–5 synapomorphies with those species, while exhibiting an astonishing ~30 autapomorphies – almost 10% unique, autapomorphic divergence from all other *Desmognathus*.

Fourth, they also exhibit 9.2% pairwise divergence *with each other,* in only 375bp. This seems incredibly unlikely. This would require ~10% intrapopulation divergence between only 6 individuals sampled from 2 sites, much higher than known in most vertebrates or other desmogs – e.g., ~4–5% across all *D. aeneus* (Pyron et al. 2024; Mol. Ecol.).

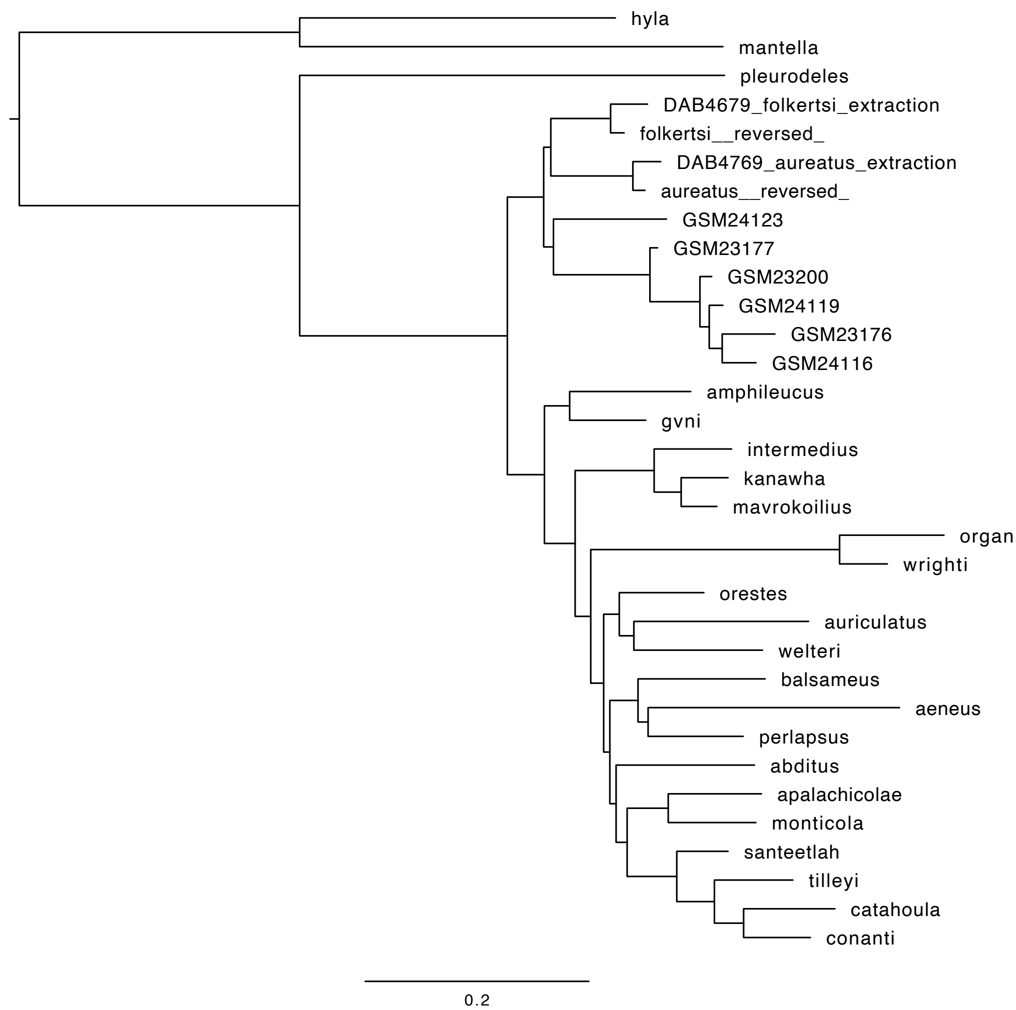
Fifth, when BLASTed, their highest match is 84% similarity to *D. folkertsi* and 81–83% to *D. aureatus,* which would be exceptional amounts of pairwise divergence between sister species. The entire alignment of the other 22 *Desmognathus* species only has 13.7% pairwise divergence overall. The next-highest would be ~11% between *D. valentinei* and *D. pascagoula*. However, the Cades Cove samples also have 81% similarity to *Hyla* (OP344519.1)*, Mantella* (AY263302.1)*,* and *Pleurodeles* (DQ821206.1).

Making a quick ML tree with all of those sequences places the aureatus/folkertsi/Cades group outside of all *Desmognathus,* while removing them yields a monophyletic Nantahala clade nested within *Desmognathus*. The extreme branch lengths of the Cades samples are also clearly visible, greater than most inter-specific branches in *Desmognathus*.

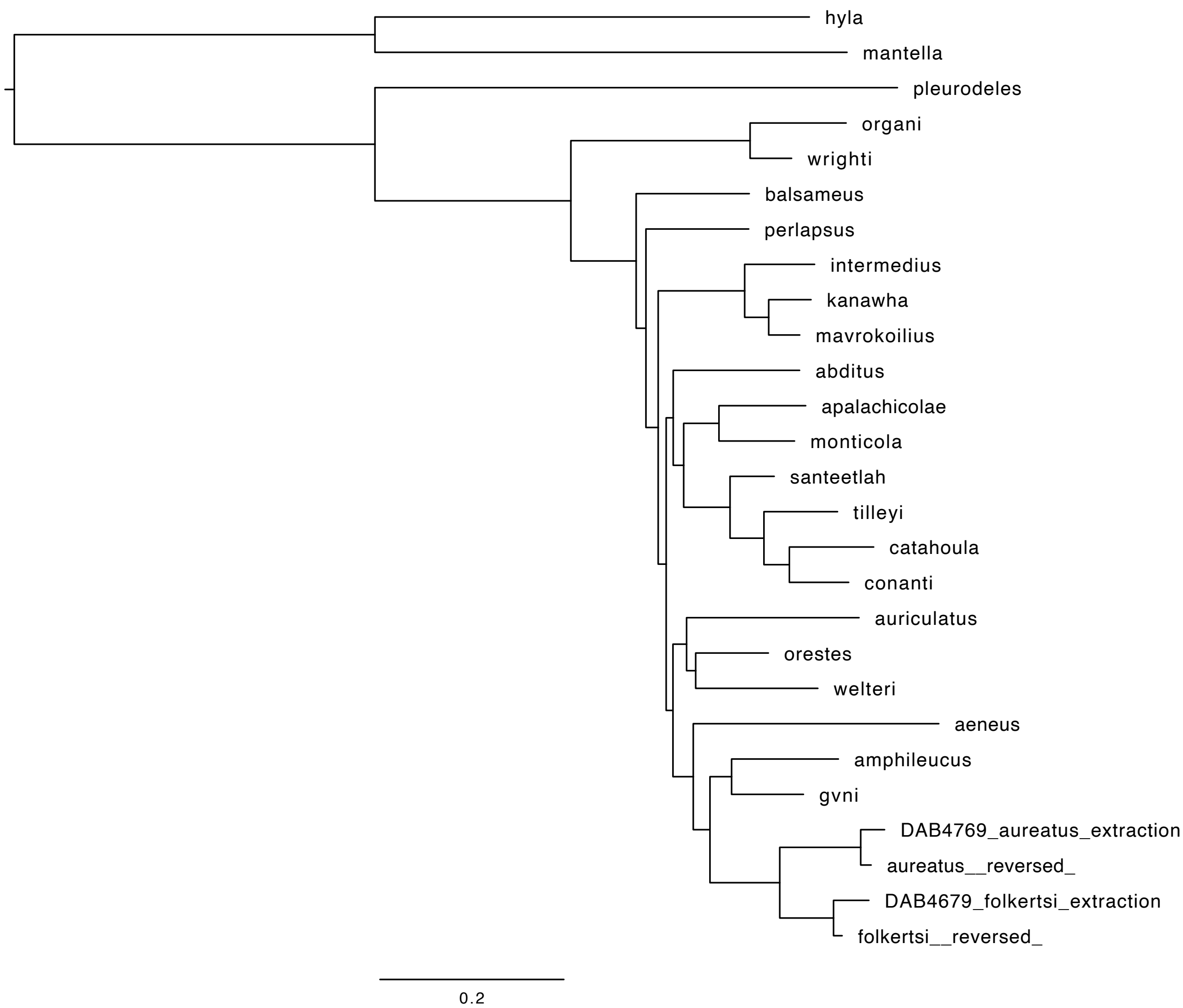
I can offer at least one explanation. What the authors hint at but don't discuss directly is that the mitochondrial sister relationship between Nantahala and Pisgah – which is falsified by their distant positions in phylogenomic datasets – is thought to be the result of a deep-time reticulation and mitochondrial genome capture between the Nantahala and Pisgah lineages prior to the diversification of the extant species (Pyron et al. 2020, 2022, 2025). **I think that the authors might have sequenced a combination of real**

mitochondria, and pseudogenic nuclear-mitochondrial insertions from this reticulation. This would explain the exceptional divergence and lack of coding.

While this does not negate the value of their data for the intended purpose of mitochondrially genotyping sites for species occupancy, it requires a much greater explanation before we can trust both the topology and presence of a “new” lineage.



Tree WITH the Cades samples – sister to ALL *Desmognathus!*



Tree WITHOUT the Cades samples – nested monophyletic Nantahala clade!

Alignment showing 10bp indel and at least 6 autapomorphies in only 65bp of sequence, along with at least 7 differences from *aureatus* + *folkertsi*