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Original Article

Widespread reticulate evolution in an adaptive radiation

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Abstract

A fundamental assumption of evolutionary biology is that phylogeny follows a bifurcating process. However, hybrid speciation and introgression are becoming more widely documented in many groups. Hybrid inference studies have been historically limited to small sets of taxa, while exploration of the prevalence and trends of reticulation at deep time scales remains unexplored. We study the evolutionary history of an adaptive radiation of 109 gemsnakes in Madagascar (Pseudoxyrhophiinae) to identify potential instances of introgression. Using several network inference methods, we find 12 reticulation events within the 22-million-year evolutionary history of gemsnakes, producing 28% of the diversity for the group, including one reticulation that resulted in the diversification of an 18 species radiation. These reticulations are found at nodes with high gene tree discordance and occurred among parental lineages distributed along a north-south axis that share similar ecologies. Younger hybrids occupy intermediate contact zones between the parent lineages showing that post-speciation dispersal in this group has not eroded the spatial signatures of introgression. Reticulations accumulated consistently over time, despite drops in overall speciation rates during the Pleistocene. This suggests that while bifurcating speciation rates may decline as the result of species accumulation and environmental change, speciation by hybridization may be more robust to these processes.

Keywords: hybridization, introgression, adaptive radiation, networks, snakes

It is generally assumed that evolution follows a bifurcating process; species are formed by the splitting of one lineage into two. This process is represented on a phylogenetic tree, showing a distinct branching pattern with measurable distances between speciation events or nodes. However, gene flow during speciation is common, not only between recently diverged extant species, but also at more ancient timescales. This ancient gene flow, referred to as deep-time reticulation occurs below the level of terminal leaves. Accounting for these events changes the structure of a phylogeny from a bifurcating graph to a network of relatedness, representing both divergent as well as reticulating speciation. Introgression has been detected in many groups, including humans (Dannemann et al., 2017; Storz & Signore, 2021), plants (Stone & Wolfe, 2021; Suarez-Gonzalez et al., 2018), fish (Bolstad et al., 2021; Olave & Meyer, 2020), insects (Pardo-Diaz et al., 2012; Suvorov et al., 2022b), and in many squamate groups such as varanids (Pavón-Vázquez et al., 2021), pit vipers (Mason et al., 2019), and kingsnakes (Burbrink & Gehara, 2018).

Given the prevalence of reticulation across the tree of life, introgression necessarily contributes to diversity. In addition, hybridization may be a mechanism that promotes speciation and accelerates diversification (Abbott et al., 2013; Grant & Grant, 2019). There are several genetic factors affecting hybridization, including genome structure and functional similarity in parents, genomic purging of deleterious introgressive regions with recombination, and levels of genetic load in populations (Moran et al., 2021). Successful introgression introduces new allelic combinations into populations that allow hybrids to persist and survive in potentially novel niches thus driving further speciation (Mallet, 2007; Richards et al., 2021; Schmickl & Yant, 2021). In some cases, hybrids have outcompeted their parent species or invaded extreme niche regions (Rieseberg et al., 2003; Sartor et al., 2021). Adaptive introgression has led to hypoxia adaptations in Tibetan mastiffs and humans (Storz & Signore, 2021), insecticide resistance in mosquitos (Norris et al., 2015), and pollution resistance in killifish (Oziolor et al., 2019). Introgression has also been shown to promote adaptive radiations (Barrier et al, 1999; Meier et al., 2017; Wu et al., 2018).

There are several factors that affect when and where hybridization occurs. Environmental disturbance has been shown to play a role in several hybrid speciation events, with signal of introgression increasing in regions with more dynamic climates (Bettles, 2004; Singhal et al., 2021; Wayne & Jenks, 1991). In a dynamic climate, disappearing geographic barriers bring allopatric species into contact, providing zones for possible hybridization (Pavón-Vázquez et al., 2021; Rhymer et al., 1994). Range contraction can also prevent hybrids from interbreeding with their parent species, driving speciation (Nolte & Tautz, 2010). Additionally, adaptation to extreme environments can increase resilience to

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environmental stressors (Czypionka et al., 2012; Rieseberg et al., 2003).

Like typical speciation processes, without environmental or other background effects, increasing numbers of species likely increases the number of hybridization events. This could be especially true for hybrid speciation in groups with overlapping ranges, as species are more likely to come into contact and hybridize. For example, taxa that diverge by ecological speciation are not spatially isolated and continue sharing genes (Nunes et al., 2022). Through the history of any clade, it may be expected that reticulations accumulate linearly on a logarithmic scale, with waiting times to the next reticulation following an exponential distribution. If ancient gene flow is less common than gene flow at terminal edges, we expect hybridization events to be concentrated in recent times and predominately among sister species pairs, with ancient hybridization events undetectable. The literature is typically biased toward these recent-time reticulations given limitations in methods requiring analyses of smaller clades. However, several instances of deeper time (>10 million years ago) introgression events are emerging (MacGuigan & Near, 2019; Singhal et al., 2021; Surovov et al., 2022a). Analyses of larger or more deeply diverged clades can identify older reticulations. At a macroevolutionary level, ecological and biogeographical trends and mechanisms for hybridization can be explored.

The Madagascar Gemsnakes (Pseudoxyrhophiidae; Lamprophiidae), exhibit a classic radiation for exploring prevalence and trends of hybridization. This group is composed of 109 morphologically diverse snakes in 17 genera occurring in Madagascar. Collectively they occupy all five distinct ecoregions of Madagascar and are adapted to several unique ecotypes: arboreal, terrestrial, aquatic, and fossorial. The group originated in a single dispersal event from Africa in the early to mid-Miocene (between 18 and 24 million years ago). They diversified at rapid and constant rates through the Miocene and Pliocene with a marked drop in speciation during the Pleistocene, attributed to likely interspecific competition and climatic and environmental change (Burbrink et al., 2019). They are the most prolific snake radiation on the island. Other snakes on the island include the closely related *Mimophis* colubrids which originated as a second dispersal of snakes from Africa, 4 species of boids, and 12 typhlophoid species (Glaw & Vences, 2007; Ruane et al., 2015). Recent phylogenomic inference for Pseudoxyrhophiidae has found several areas of the tree to have low-supported node values. Additionally, different phylogenetic inference methods have shown alternative topologies in several regions of the tree (Burbrink et al., 2019). Weakly supported nodes could be due to poor within-gene phylogenetic signal (i.e., gene tree relationships cannot be confidently inferred), which would directly affect species-tree resolution. Alternatively, this could represent introgression, which if not explicitly tested could result in poor support for a bifurcating tree (Morales-Briones et al., 2021).

Madagascar is ideal for studies of speciation due to its exceptional climatic variation, regional endemism, and cryptic reptile parapatric speciation patterns, which provide conditions to promote ecological speciation and hybridization during climate change (Florio et al., 2012; Raxworthy et al., 2007, 2008a). The island has experienced considerable environmental change, particularly categorized in the Quaternary (Kuhn et al. 2022, Pearson & Raxworthy, 2009; Raxworthy et al., 2008b). Glacial cycles in the temperate regions of the world have contributed to changing climatic conditions on the island over the past several million years (Vences et al., 2009; Wilmé et al., 2006). These changing dynamics can affect gene flow and thus probabilities of successful hybridization and speciation, particularly in the Pleistocene onward, when these periods of environmental change are affecting the region.

It is necessary to develop a comprehensive pipeline to identify reticulations in large phylogenies. Computational limitations of network inference require splitting the tree into several testable clades, with careful attention to possible gene flow between those clades. Several methods use discordance patterns to infer a network structure for a phylogeny, each with differing assumptions, inputs, or returns (Allman et al., 2019; Blischak et al., 2018; Solís-Lemus & Ané, 2016; Wen et al., 2018). For example, NANUQ, SnaQ and several other network inference methods require gene tree estimation, while HyDe, a hybrid detection program, does not. SnaQ can return several equally likely topologies, so alternative methods (i.e., NANUQ) and support metrics (i.e., a goodness of fit test) can differentiate between them. Furthermore, biogeographic evidence can provide support for reticulation (Burbrink & Gehara, 2018; Pavón-Vázquez et al., 2021). Hybrids are formed in the contact zones between two species and introgression is correlated with geographic proximity (Singhal et al., 2021). In addition to providing support for a given reticulation, the biogeography of hybrids can inform dispersal trends for the gemsnakes. If post-speciation dispersal is limited, we expect hybrids to have a range in between those of the parents, with decreasing biogeographic signal over time as a clade diversifies.

In this paper, we examine Pseudoxyrhophiidae to determine if the evolutionary history of this group is best described as either a bifurcating tree or a reticulate network. We identified nodes with high gene tree discordance to locate likely areas of reticulation and used four network inference/support methods (NANUQ, SnaQ, HyDe, Quartet Goodness of Fit Test) to infer reticulations across the phylogeny while considering the prevalence of phylogenetically uninformative genes. We hypothesize that given the rapid rate of speciation, and local areas of poorly supported topology, that this radiation may show several reticulate events which consistently accumulate over time. We also predict that reticulation events should occur in areas intermediate to parent species and among taxa showing similar environmental and ecological habits.

Methods

Data and phylogenetic analysis

We obtained 391 phased anchored hyrbid enrichment (AHE) loci for 115 species and 366 individuals of Pseudoxyrhophiidae from Burbrink et al. (2019). Gene trees were constructed in IQTree2 along with 1,000 ultrafast bootstraps (Minh et al., 2020). We inferred a species tree using ASTRAL III (Zhang et al., 2018). Using fossil outgroups for which AHE data were available, the phylogeny was dated using Tree PL, with error estimated using bootstrapped gene trees (Smith & O'Meara, 2012; see Burbrink et al., 2019 for additional details on divergence dating in this group). Given computational limitations in the network inference analyses used in this study, we separated the tree into monophyletic groups by splitting the set of 109 core taxa into 10 clades spanning 5–18 taxa each. Clade choices were made based on high nodal support in the ASTRAL tree (from quadripartition support) as well

as strong monophyletic support across gene trees visualized in DiscoVista (Sayyari et al., 2018). This allowed us to rule out between-clade reticulations. The groups consisted of (1) *Lycodryas*, (2) *Micropisthodon*, *Ithycyphus,* and *Langaha*, (3) *Phisalixella*, (4) *Madagascarophis*, (5) *Compsophis* and *Alluaudina*, (6) *Thamnosophis* and *Dromicodryas*, (7) *Liophidium*, (8) *Heteroliodon* and *Pseudoxyrhopus*, (9) *Liopholidophis*, (10) *Parastenophis* and *Leioheterodon*. Additionally, we sampled each clade four times to construct sets of 11 species, with one representative from each clade and the monotypic *Elapotinus* species, to identify reticulations at the base of the tree. Gene tree concordance factors were calculated for all quartet taxon sets in each analysis, to the individual level, using the function quartetTable() from MSCquartets 1.0 in R (Rhodes et al., 2021).

Gene tree discordance

We identified and visualized gene tree discordance and assessed how well the gene trees matched the ASTRAL-III species tree. Alternative species trees (ASTRAL-II, RaxML, IQtree, SVDQuartets) were obtained from Burbrink et al. (2019) to assess discrepancies between inference methods.

To detect alternative gene tree topologies and areas of conflict across the entire tree, we used PhyParts (Smith et al., 2015). Areas of the tree that had a high proportion of informative gene trees, but with many supporting topologies alternative to the species tree, may represent reticulations. Alternatively, areas of the tree with a high proportion of uninformative genes (i.e., loci that were unable to determine the sister relationships between clades) may not be able to detect reticulate evolution due to low power and confounding signal caused by a poorly chosen pool of genes (Morales-Briones et al., 2021). Reticulations inferred in these areas with low phylogenetic signal (more than 15% uninformative genes) were reassessed by either removing the uninformative genes or removing taxa that created uninformative nodes and performing the network analysis again.

Network analysis

We used SnaQ (Solís-Lemus & Ané, 2016), implemented in PhyloNetworks package (Solís-Lemus et al., 2017) of Julia (Bezanson et al., 2017), to infer network structure on a phylogeny, which includes estimation of genomic contribution of parents to hybrids (γ), location of reticulations, and the pseudolikelihood for the given topology. SnaQ searches quartet gene trees to find concordance and estimates likelihood for the given four-taxon unrooted topology, taking a bifurcating or cyclic structure. It builds the most likely species network from that set of quartets and assigns a pseudolikelihood value to the overall network structure. Networks were estimated for 0–5 reticulations for our clades ranging from 5–18 taxa; though we limited our study to reticulations in which genomic contribution of both parent lineages was greater than ten percent. To estimate a species level network, individual concordance factors were averaged to obtain species-level estimates using the function *mapAllelesCFtable* in the PhyloNetworks package (Solís-Lemus et al., 2017). Pseudolikelihoods were calculated using the function *topologyMaxQPseudolik!* in PhyloNetworks which optimizes the branch lengths and gamma values (percent genomic contribution) along the tree. Use of this function allowed us to compare pseudolikelihoods of trees output by programs other than SnaQ (i.e., NANUQ). The optimal number of reticulations was estimated by assessing the change in pseudologlikelihood values with increasing number of reticulations. Slope heuristics for change in pseudologlikelihood were calculated; a change in likelihood of less than or equal to two indicated that the optimal number of reticulations had been reached.

Additional support for non-bifurcating network topologies was obtained by performing SnaQ on the set of bootstrapped gene trees when computationally feasible. Bootstrap gene trees were pruned to represent one individual per species. We used 10–100 bootstrap replicate trees as input to bootsnaq implemented in PhyloNetworks, depending on computational limitations for each clade. This calculated the network structure for each replicate for one to five reticulations. Networks for each bootstrap were stripped of reticulations in which genomic contribution from either parent was less than 10%. Bootstrap support was calculated for the best topology, including support for the hybrid and parent nodes.

To visualize introgressive gene flow between all taxa, we ran NANUQ, a multispecies coalescent model for detecting reticulations. NANUQ assessed gene concordance for all quartet sets of individuals to build a splits graph representing the relationships across the whole network (Allman et al., 2019). While accounting for ILS, the program assesses the probability of quartets having tree structure and the probability of quartets having a star structure. Quartets that are likely not a tree and not unresolved (a star) represent a cyclic structure. Statistical support values to determine if the quartet was a tree, cycle, or star were chosen based on the groupings of all quartet probabilities such that apparent groupings fell under the same distinction (e.g., all quartets involving individuals of taxa a,b,c, and d are either all a tree/all not a tree; all a star/all not a star). This output was visualized on a ternary plot output by the function NANUQ(), implemented in MSCQuartets 1.0 (Rhodes et al., 2021). The relationships among all quartets were drawn onto a single network as a splits graph, created in SplitsTree4 (Huson & Bryant, 2005). Previous views of splits graphs showed signatures of gene flow among all individuals but could not be translated into a rooted network with discernible cycles (Huson et al., 2010). Allman et al. (2019) have outlined darting patterns that can be distinctly translated into typical reticulate cycles on a rooted tree, like those output by SnaQ. For the individual-level trees, NANUQ was unable to show any of these described darting patterns, so we subsampled the unrooted trees to have one individual per species to better see the structure of the species-level phylogeny.

Given the networks output by SnaQ and NANUQ, we examined the goodness of fit of these topologies to the data. This was done using the function *quarnetGoFtest!()* in QuartetNetworkGoodnessFit package (Cai & Ané, 2021) implemented in Julia. This program identified quartets in the data that fit the network topology. More likely topologies have higher *p*-values, corresponding to a high proportion of well-fit quartet trees.

Finally, to validate the best-found topology using a method that did not rely on gene tree estimation, we used HyDe (Blischak et al., 2018). HyDe analyzes site variation among species triplets to identify hybrid–parent relationships and when using concatenated genes, has been shown to recover the correct topology with higher accuracy than the D-statistic (Kong & Kubatko, 2021). We ran HyDe on the set of 371 concatenated anchored loci and identified the proportion of triplets (out of all possible triplets for a reticulation) that were significant ($p < .05$ and gamma values between 0 and 1).

Ecological and biogeographic influence on hybridization

We also examined the biogeographic locations and ecological traits associated with the parent-hybrid regimes to support our findings. Occurrence point localities for each individual and scoring of species dentition and habitat type were obtained from Burbrink et al. (2019). Additional data on the range for *Pseudoxyrhopus oblectator* was derived from the IUCN Red List (Vences et al., 2011). Localities were mapped onto the ecoregions of Madagascar using QGIS (2021). We identified range overlap among parent and hybrid ranges to examine if the hybrid range was intermediate to that of the range of the parent taxa. Here, we defined intermediate hybrid individuals as those that existed between the northern-most locality of the northern parent and the southern-most locality of the southern parent. We classified hybrid species as intermediate if at least 85% of hybrid individuals fell in this range. We also calculated the minimum distance of the hybrid from each parent population by finding the two closest occurrence points (of the hybrid and parent) and calculating the Euclidean distance between them. We scored dentition type and habitat to identify similarities among the parent and hybrid lineages (or extant descendants of the parent or hybrid lineage).

Trends in hybridization through time

We estimated age of reticulations using the dated bifurcating tree, identifying periods of time in which the parents coexisted and could produce the reticulate offspring. The maximum age of reticulation was defined as the timing of origin of the most recent parent while the minimum age was the youngest time at which both parent lineages existed. Timing of reticulation was taken as the midpoint of this age interval. These dates were used to create a reticulation through time plot, showing the accumulation in hybridization events throughout the clade's history on a semi-log scale. We estimated the rate of reticulation (assuming constant rate) as the slope of the line joining the endpoints of this step function (Supplementary Figure S5).

We binned the ages of each terminal taxon whose immediate ancestors were not involved in a reticulation by whether there was a reticulation involving this taxon (i.e., the taxa is a parent or hybrid) or not. A simple *t*-test allowed us to identify if reticulating lineages were on average younger than non-reticulating lineages at the level of terminal taxon. The phylogenetic distance between parent lineages was also calculated as the length (in millions of years) from the parents to the most recent common ancestor. Because the parent could have contributed genes to the hybrid at any point along its branch, an interval of possible distances was calculated. Additionally, gene tree discordance was compared to node age and distance to the most recent ancestor using a linear regression model to identify if introgression scaled with time. Nodes were classified as reticulation nodes if they were within the distance between the hybrid origination node and the common ancestor of the parents and the node propagated at least one hybrid or parent individual. To examine if reticulating nodes had higher discordance, a density plot for the discordance proportion (excluding uninformative genes) was drawn for reticulating and non-reticulating nodes. A bootstrap statistical test was performed, with 10,000 pairwise points drawn from the distributions of reticulating and non-reticulating nodes. The proportion of draws with higher reticulating node discordance was calculated.

Results

Phylogenetic analysis and phylogenetic tree discordance

The ASTRAL III species tree had high support for all nodes and was similar to the ASTRAL II topology of Burbrink et al. (2019) except for two instances: *Thamnosophis martae* was sister to *T. sp A, T. mavotenda* and *T. sp B* in the ASTRAL-II tree; now, *Thamnosophis sp A* is sister to that *T. martae, T. mavotenda* and *T. sp B, (*matching the IQTREE and RaxML trees); and *Lycodryas granuliceps* was sister to *L. pseudogranuliceps* in the II topology; now, *Lycodryas* sp G is sister to *L. pseudogranuliceps*. The SVD quartet tree shows *Micropisthodon* as the outgroup to *Langaha* and *Ithycyphus* while the other tree inferences (ASTRAL, RaxML, IQtree) place it as sister to *Ithycyphus*. The SVD quartet tree switched the positions of *Thamnosophis epistibes* and the undescribed *T. cf. epistibes*. and the positions of *Phisalixella sp B* and *Phisalixella arctifasciata*. Further, the *Ithycyphus, Langaha, Micropisthodon* clade had low resolution, with about a third of gene trees supporting each of the possible phylogenetic combinations of genera.

The ASTRAL-III tree was used in all analyses as the bifurcating species tree. TreePL recovers the age of the family to be 22.17 million years old, with node dates consistent with Burbrink et al. (2019).

Gene tree discordance

Three nodes of the tree had a substantial proportion (more than 15%) of uninformative genes: (1) *Liopholidophis baderi* and *Liopholidophis oligolepis*, (2) *Madagascarophis colubrinus B* and *Madagascarophis colubrinus C*), and (3) *Pseudoxyrhopus quinquelineatus A* and *Pseudoxyrhopus quinquelineatus B* (Figure 1). Network inference was performed on the entire dataset in these areas. Additionally, one of two approaches were taken in these regions to improve the accuracy of results. In instances where more than 45% of gene trees were informative (case 2), the uninformative gene trees were pruned from the analysis so reticulations could be detected among all taxa for the group more accurately. Alternatively (cases 1 and 3), taxon sampling was performed to remove progeny from the uninformative node (for these sister species trios) so that all gene trees could be used in the analysis.

All genera in the tree contained at least one node with a significant proportion of discordant genes (>40%) except for *Leioheterodon*, *Parastenophis*, *Dromicodryas*, and *Alluaudina*, the least diverse clades here. Several deeper nodes also displayed high levels of discordance such as those uniting *Leioheterodon* and *Parastenophis*, the arboreal *Compsophis* and *Alluaudina* with the terrestrial gemsnakes, the node joining *Pseudoxyrhopus* and *Heteroliodon* with *Liopholidophis* and *Liophidium* and the node uniting the three major clades of gemsnakes (Figure 1).

Clades chosen for this study were demonstrated to be monophyletic according to the Gene Tree Discordance analysis, with a high proportion of genes (more than 75%) showing monophyly for almost all groups (Figure 1). Approximately half of the gene trees did not support monophyly for at least one individual of the clade comprising *Pseudoxyrhopus* and *Heteroliodon*. Monophyly for *Pseudoxyrhopus* and *Heteroliodon* as separate clades was also poorly supported, as well as monophyly for a large clade comprising the

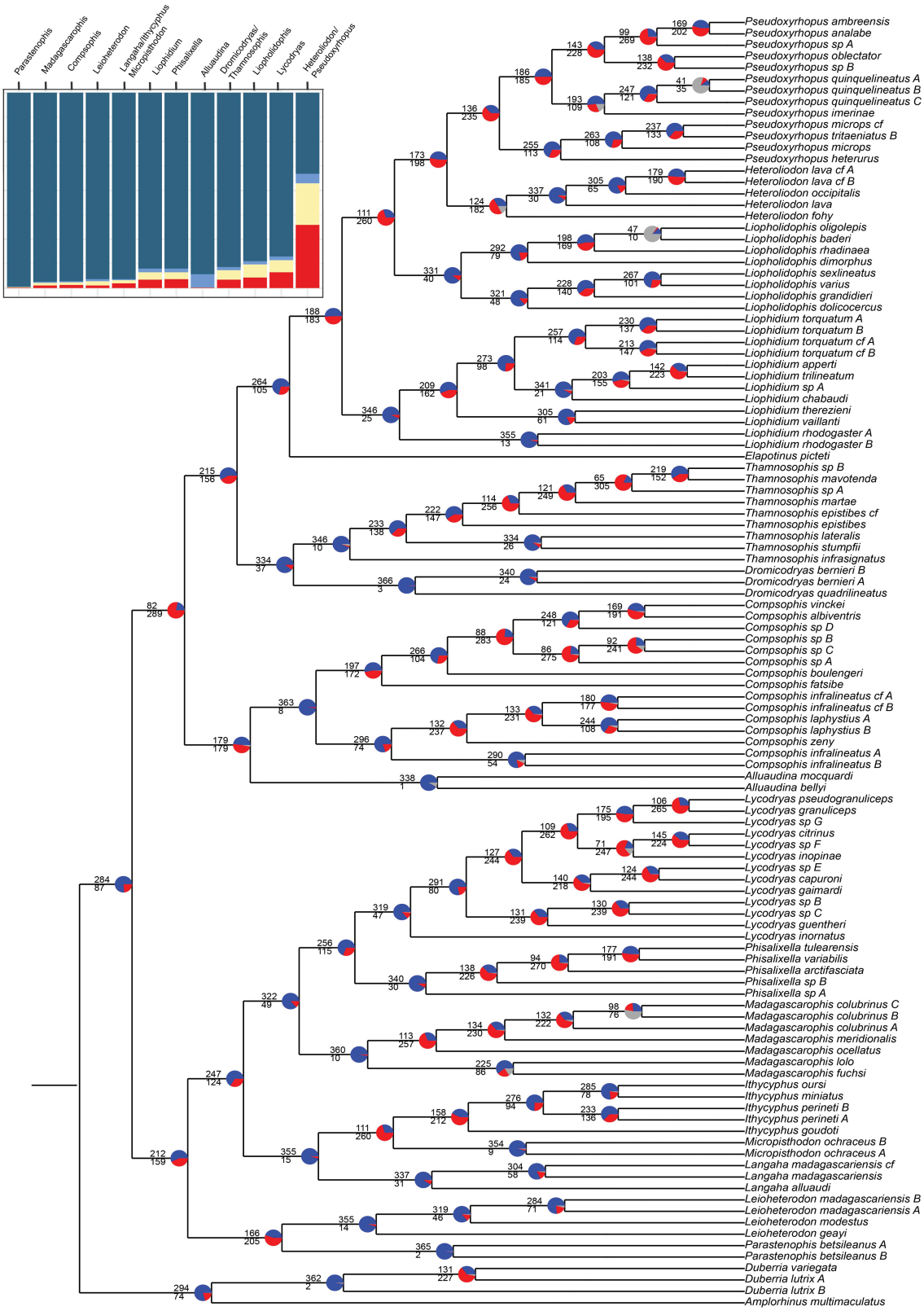

Figure 1. Phylogeny of Pseudoxyrhophiidae, depicting gene tree conflict. Blue shows the proportion of gene trees matching the ASTRAL-III topology, red shows the proportion matching alternative topologies, gray is the proportion of uninformative genes. Values above each branch adjacent to the pie chart represent the number of gene trees that match the species tree, below are those that match alternative topologies. The upper left graph depicts gene tree support for the monophyletic clades. Dark blue indicates the proportion of gene trees with strong support for monophyly, light blue is weakly supported. Yellow indicates that monophyly is weakly rejected and red is the proportion that strongly reject monophyly for the group.

genera *Pseudoxyrhopus*, *Heteroliodon*, and the sister genus *Liopholidophis* (Supplementary Figure S1). The clade groupings of *Parastenophis* and *Leioheterodon* and *Compsophis* and *Allaudina* also displayed low monophyletic support. Enhanced discordance likely exists in these two groupings of deeply diverged clades with only a few extant species because

these relationships are the most difficult to resolve with these genetic data (Whitfield & Lockhart, 2007). When we separated these deeply diverged genera, there was greater support for monophyly (>75%; Supplementary Figure S1). This discordance was thus not likely to be an indication of reticulate evolution occurring with individuals outside these clades.

Reticulation events

Both SNAQ and NANUQ predicted bifurcating clades for *Liophidium*, *Liopholidophis*, and *Parastenophis/Leioheterodon* for 71%, 75%, and 78% of the data, respectively (Figure 2).

Liopholidophis had a poorly supported node, with 85% of gene trees showing polytomy between *L. oligolepis*, *L. baderi*, and *L. rhadinaea*, which affected the ability for the data to infer any topology. Since removal of these uninformative genes would create a dataset too small for this study, *L. oligolepis* was randomly chosen to be removed from the network analysis. In the complete analysis, SnaQ did not infer *L. oligolepis* to be involved in a reticulation (a bifurcating topology was best supported), therefore, removing it from the analysis would not negatively affect the results. Removal of this taxon improved the score of the bifurcating tree from supporting 17% to 75% of the data (Supplementary Table SN15).

The genus *Lycodryas* showed significant support across all methods for a network with two reticulations (Figure 2). Six networks had equivalent pseudolikelihood and goodness of fit support, with two involving the same sets of taxa. Of these taxa, the most frequent hybrids are: *L. sp F* (this reticulation shown in 4/6 topologies) and *L. pseudogranuliceps* (shown in 4/6 topologies; Supplementary Figure SN1). NANUQ showed support for *L. sp F* as hybrid, but switched the relationship of the other, depicting *L. sp G* as the hybrid (Supplementary Figure SN2). This discrepancy was consistent with the discordance between the ASTRAL II and III topologies. The configuration shown in SnaQ (-pseudologlik = 187.1) is more likely than the NANUQ configuration (-pseudologlik = 231.2), which fit these data more poorly (Supplementary Table SN1). Thus, the inferred network depicts *L. citrinus* (γ = 0.84) and *L. inopinae* (γ = 0.16) forming *L. sp F* and *L. granuliceps* (γ = 0.66) and *L. sp G* (γ = 0.34) forming *L. pseudogranuliceps* (p = .89). Further, *L. sp G*, the minor parent, resides in the southern areas of the island, *L. granuliceps,* the major parent, in the northern end, and *L. pseudogranuliceps*, the hybrid, connects these two populations, further supporting the SnaQ configuration. The same range scenario (parents at north and south and hybrid central) was seen for the *species F* reticulation (Supplementary Figure SN4). Several different reticulate configurations were shown in bootstrap pseudoreplicates, with the reticulation for *species F* appearing 13% of the time and the *L. pseudogranuliceps* reticulation occurring 20% of the time across 97 trees (Supplementary Table SN2). Both of these triplets were identified by HyDe.

Phisalixella showed discordance among all members of the clade; this was the result of *species B* changing position across gene trees (Figure 1). Additionally, there were discrepancies about the placement of *species B* in the original study (Burbrink et al., 2019). The best supported network by SnaQ and the goodness of fit test (p = 0.76) involved an ancestor to the clade contributing 39% of the genome of *species B* (Supplementary Table SN3). *Phisalixella sp B* lies in the northernmost part of the island, overlapping in range with none of the extant members of *Phisalixella* (Supplementary Figure SN6). The NANUQ tree uniquely showed gene flow between all members of the clade (Supplementary Figure SN7). NANUQ was unable to depict a cycle where all members of a reticulation are not sampled. Therefore, this network was consistent with the network found in SnaQ. This diversification scenario was matched by 46.4% of bootstrap trees (n = 196; Supplementary Table SN4). Given that this network involved an extinct or unsampled taxon, we could not test it with HyDe.

The genus *Madagascarophis* showed a high proportion of uninformative genes (53%) at the node uniting *M. colubrinus B* and *M. colubrinus C* (Figure 1). Analysis on all gene trees found no trees with high support (p < .15) and SnaQ was unable to identify a best topology, only able to distinguish that at least one reticulation was present (Supplementary Table SN5). Subsampling the gene trees to only include the 174 genes that were informative for that node improved the results slightly. The best tree inferred a reticulation between *M. colubrinus A* (γ = 0.84) and ancestor to *M. fucshi* and *M. lolo* (γ = 0.16) yielding the ancestor to *M. colubrinus B* and *M. colubrinus C*. Bootstraps of trees for the entire dataset (n = 450) and trees for the uninformative dataset (n = 299) recovered this reticulation in 71% and 79% of the trees, respectively (Supplementary Table SN6). Biogeographically, *M. fuchsi* and *M. lolo* are currently confined to the northernmost tip of the island, along with *M. colubrinus B* and *C* which have a slightly larger range. *Madagascarophis colubrinus A* is found south of this area, not overlapping with the other species (Supplementary Figure SN11). The network containing this reticulation described 33% of the informative data, which improved from describing 14% of the entire dataset. Though all two-hybrid networks found in SnaQ were not supported by the data, this small percentage may indicate the presence of a more complex network in which a member of the reticulation was part of another hybridization. NANUQ supports this; the best topology shows gene flow between *M. fucshi/lolo* and *M. colubrinus B/C* but does not display a cycle that would be expected if this were the true topology (Supplementary Figure SN12). Additionally, HyDe displayed several significant hybrid pathways that were inconsistent with each other topologically (Supplementary Table SN7).

A two-reticulation network best described the topology of the clade consisting of the genera *Langaha*, *Ithycyphus*, and *Micropisthodon* (Figure 2). NANUQ identified the best network as including all species of *Langaha* and *Ithycyphus* forming *Micropisthodon*. This reticulation was poorly supported by the data. However, the network involving *Langaha* (γ = 0.84) and *Ithycyphus* without *I. goudoti* (γ = 0.16) forming the *Micropisthodon* clade was found to fit the data well (Supplementary Table SN6). This reticulation is consistent with issues of method discordance identified in Burbrink et al. (2019). Extant members of the clades were found across the island, with the rare *Micropisthodon* occurring in a few localities along the eastern side (Supplementary Figure SN16). An additional reticulation appeared in 44% of bootstrap replicates (n = 152), involving *I. perineti B* (γ = 0.9) and the ancestor to *I. miniatus* and *I. oursi* (γ = 0.1) forming *I. perineti A*. Together, these reticulations accounted for 80% of the quartet data. HyDe identified the hybrid triplet for *I. perineti B* but identified none of the triplets for the generic level reticulation despite these genera having significant discordance among inferred phylogenies and gene trees.

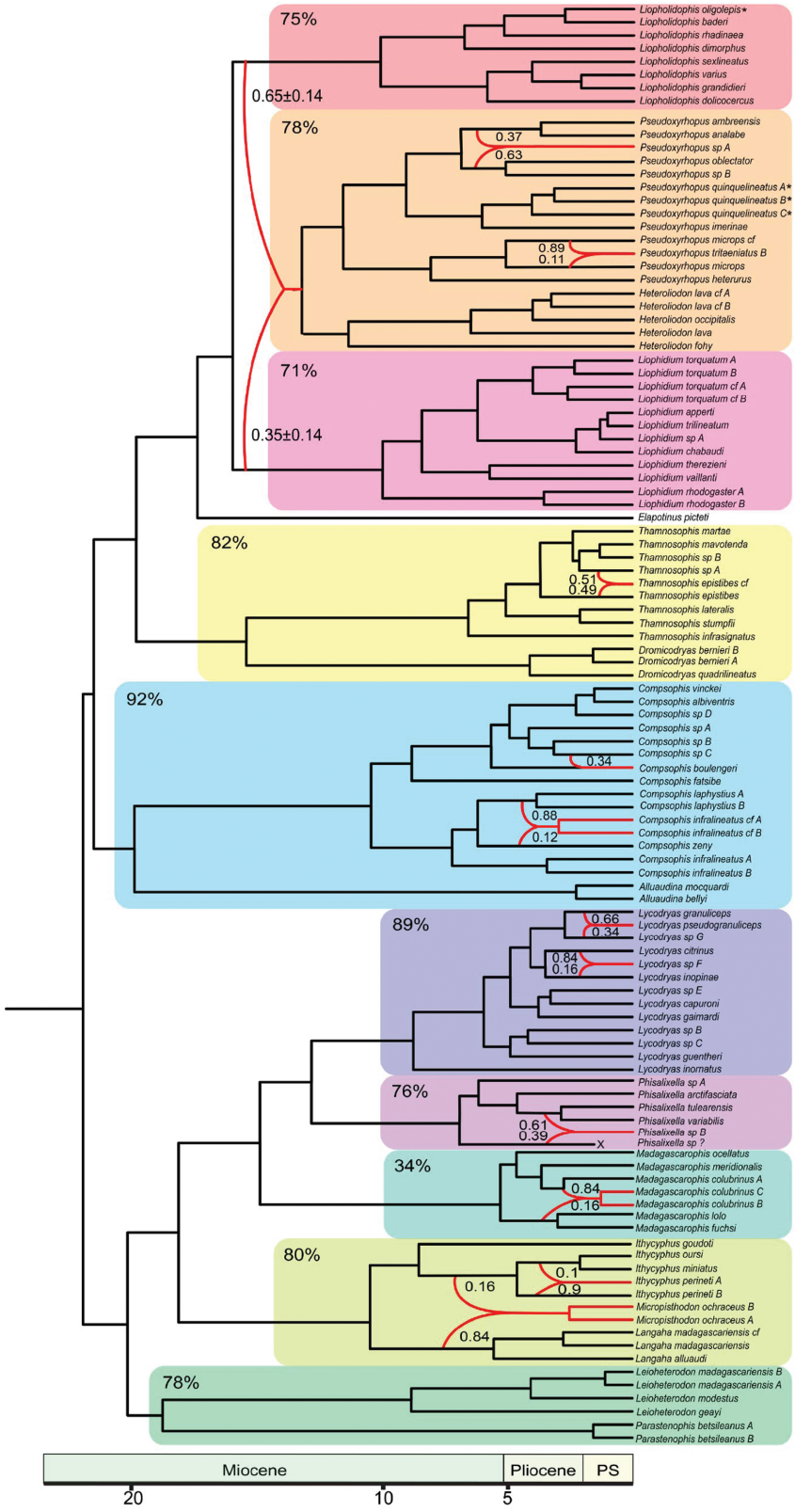

Figure 2. Dated Phylogenetic Network for the Malagasy Gemsnakes. Reticulations are depicted as red lines connecting parent lineages to the hybrid taxon or taxa. Proportion of genomic contribution from each parent lineage is denoted. Explicitly tested clade groupings are highlighted. Percent of quartet data supporting the given topology is shown for each clade. Asterisks indicate taxa that were excluded from network analyses because of uninformative nodes. PS = Pleistocene.

Two reticulations were supported for *Compsophis*. One involving *C. sp C* contributing genes (γ = 0.34) to present day *C. boulengeri*. The present-day lineages overlap in range. The second reticulation involved *C. zeny* (γ = 0.16) and the ancestor to two species of *C. laphystius* (γ = 0.84) yielding two species of *C. cf. infralineatus*. This network structure

explained 92% of the quartet data for this tree and can be seen in the NANUQ tree, with gene flow between *boulengeri* and *sp C* shown (Supplementary Figure SN20). *Compsophis cf. infralineatus* and *C. laphystius* are found across the entire eastern side of the island with *C. zeny* confined to the south (Supplementary Figure SN21). All four triplets were identified by HyDe for the hybrid *C. cf. infralineatus*; a triplet for *C. boulengeri* could not be identified given that this is unidirectional introgression with only one parent.

The genus *Thamnosophis* had one reticulation among its members, *T. epistibes* ($\gamma = 0.49$) and *T. sp A* ($\gamma = 0.51$) forming *T. cf. epistibes* ($p = .82$). *Thamnosophis epistibes* and *T. sp A* are found along the eastern edge of the island with *T. sp A* confined to the north. The hybrid, *T. cf. epistibes*, also lies in the north, overlapping with both parents' ranges (Supplementary Figure SN26). This was the most common reticulation found among bootstrap pseudoreplicates, with 21% of replicates displaying this hybridization (Supplementary Table SN11). NANUQ inferred this reticulation as well, which is consistent with discrepancies in bifurcating tree inference methods (Burbrink et al., 2019). The triplet was identified by HyDe.

The genus *Pseudoxyrhopus* had one uninformative node involving three species of *P. quinquelineatus*. Network analysis of all genes did not reveal a reticulation involving any of these species. These species were removed from the analysis and the reticulation study was rerun. The best supported topology was a network with two reticulations, one involving *P. cf. microps* ($\gamma = 0.83$) and *P. microps* ($\gamma = 0.17$) forming *P. tritaeniatus*. The other reticulation had two indiscernible scenarios, one involving *P. analabe* ($\gamma = 0.3$) and ancestor to *P. oblectator* and *P. sp B* ($\gamma = 0.7$) yielding *P. sp A* and the other involving ancestor to *P. ambreensis* and *P. analabe* ($\gamma = 0.67$) and ancestor to *P. oblectator* and *P. sp B* ($\gamma = 0.33$) yielding *P. sp A* with approximately 78% of quartets supporting both scenarios. However, the NANUQ analysis agreed with the latter reticulation scenario (Supplementary Figure SN30). HyDe showed all triplets involved in these reticulations (1 and triplets, respectively) as being significant. The distribution for *P. sp A* lies in the central eastern side of the island, between the ranges for both sets of parent lineages. *Pseudoxyrhopus tritaeniatus* lies between the ranges of its parental lineages as well, which are found in the eastern humid side of the island (Supplementary Figure SN31).

Analysis of the subsampled backbone of the tree, comprising one representative species per clade, found one ancient reticulation occurring between genera *Liopholidophis* and *Liophidium* yielding an 18 species radiation comprising *Pseudoxyrhopus* and *Heteroliodon*. This is consistent with the analysis of monophyly which showed very poor support for the clade of *Pseudoxyrhopus* and *Heteroliodon*. In all trials, NANUQ showed clear gene flow between members of *Liopholidophis*, *Liophidium*, and the *Pseudoxyrhopus/Heteroliodon* clade (Supplementary Figure SN33). SnaQ and the goodness of fit test supported networks involving *Liopholidophis* (with per-trial genomic contributions: 81, 81, 70, and 60%) and *Liophidium* (19, 19, 30, and 40%, respectively) yielding *Pseudoxyrhopus* and *Heteroliodon* in all trials (Supplementary Table SN14). HyDe identified 896 significant triplets out of the 1281 involved in this reticulation (70%); all triplets involving *Liopholidophis baderi* were not significant. The placement of this species was shown to have poor phylogenetic signal (Figure 1), therefore we excluded it from analyses, improving the result to 895/1,120 triplets (80%). This is the oldest reticulation detected, occurring 14.7 (±1.42) million years ago.

Temporal and ecological patterns of reticulations

Reticulation dates ranged from 14.7 to 1.1 million years ago (Figure 2) having more introgressions occurring at younger times. We calculated an origination rate for reticulations of 0.169 per million years, assuming constant rate through time. Lineage accumulation was constant through time for the group with speciation rates dropping in the Pleistocene (Burbrink et al., 2019). Hybrid groups have the same dentition and activity pattern (Table 1) excluding *Micropisthodon* (currently considered aglyphous and nocturnal) and the parent species *Ithycyphus* and *Langaha* (both opisthoglyphous and diurnal) and *Pseudoxyrhopus* and *Heteroliodon* (nocturnal) and parent species *Liopholidophis* and *Liophidium*. However, further investigation is required to confirm dentition in the rare *Micropisthodon*. Hybrid groups also shared the same habitat (Table 1). Distribution patterns for the hybrid-parent regimes were typically North-South distributed, with hybrids occupying the shared ecoregions of the parents, and parents having shared ecoregions. Gene tree discordance did not scale linearly with age to the most recent ancestor ($p = 0.224$; $R^2 = 0.007$) or node age ($p = 0.485$; $R^2 = 0.005$; Supplementary Figure S2). Among the set of terminal taxa whose immediate ancestral lineage did not undergo reticulation, we demonstrate that on average reticulating taxa were not younger than non-reticulating taxa ($p = 0.354$; Supplementary Figure S3).

Discussion

Within a single 22-million-year adaptive radiation that produced 109 extant species, we identified 12 instances of reticulate evolution, indicating that the phylogenetic history of the Gemsnakes is best represented as a network rather than a bifurcating tree. Using a comprehensive multi-method pipeline, we uncovered that gene flow accounted for a high proportion of the biodiversity in this group and contributed to an 18-species radiation on the island. Thirty species, representing 28% of all extant taxa, are strong candidates to have had an origin involving some type of ancestral hybridization (4 of the 12 hybrid lineages further diversified to produce additional taxa). This contrasts with conventional ideas that once speciation has occurred then gene flow among taxa should be uncommon (Mayr, 1942, 2000). Reticulations are restricted to the last 15 million years with continuous accumulation through time, despite declining overall speciation rates (Figure 3). Reticulations were not clustered within particular groups or ecologies, suggesting that hybridization is an important process contributing to diversity throughout this entire radiation.

Trends of time and phylogenetic scale

Speciation with gene flow is common among many groups (Burbrink & Ruane, 2021), but gene flow between more distantly related or fully reproductively isolated species is more recently being recognized as a fundamental process in evolution. If significant gene flow does not occur at deeper time scales frequently, we would expect this gemsnake radiation to show a concentration of reticulate events at more recent times and between sister taxa. However, reticulations are present across the entire tree, with consistent accumulation over time (Figure 3). Further, our reticulations occur among more distantly related species (7/12 reticulations), as

Table 1. Ecology and behavior for parent and hybrid taxa.

Taxon	Identity	Dentition	Activity	Habitat	Ecoregion
Lycodryas citrinus	Major	O	N	Ar	D
L. sp F	Hybrid	O	N	Ar	E,H
L. inopinae	Minor	O	N	Ar	H
Lycodryas granuliceps	Major	O	N	Ar	E,H,D
L. pseudogranuliceps	Hybrid	O	N	Ar	H,D
L sp G	Minor	O	N	Ar	E,D
Madagascarophis colubrinus A	Major	O	N	G	E,H,D
M. colubrinus B	Hybrid	O	N	G	E,H
M. colubrinus C	Hybrid	O	N	G	H
M. fuchsi	Minor	O	N	G	D
M. lolo	Minor	O	N	G	D
Phisalixella tulearensis	Major	O	N	Ar	H.D
P. variabilis	Major	O	N	Ar	E,D,A
P. sp B	Hybrid	O	N	Ar	E,D
Thamnosophis sp A	Equal Parent	A	D	T	E
T. epistibes cf	Hybrid	A	D	T	E,H
T. epistibes	Equal Parent	A	D	T	E,H
Compsophis sp C	Minor	O	N	G	H
C. boulengeri	Hybrid/ Major	O	N	G	E,H
Compsophis laphysitus	Major	O	N	Ar	E,H
C. infralineaus cf	Hybrid	O	N	Ar	E
C. zeny	Minor	O	N	Ar	E,H
Ithycyphus perineti B	Major	O	D	Ar	E,H,D
I. perineti A	Hybrid	O	D	Ar	E,H,D
I. miniatus	Minor	O	D	Ar	E,D
I. oursi	Minor	O	D	Ar	E,D,A
Langaha	Major	O	D	Ar	E,H,D,A
Micropisthodon	Hybrid	A	N	Ar	E
Ithycyphus	Minor	O	D	Ar	E,H,D,A
Pseudoxyrhopus sp B	Major	A	N	T	H
P. oblectator	Major	A	N	T	E,H
P. sp A	Hybrid	A	N	T	E,H
P. ambreensis	Minor	A	N	T	E,D
P. analabe	Minor	A	N	T	E,H
Pseudoxyrhopus microps cf.	Major	A	N	T	E,H
P. tritaeniatus B	Hybrid	A	N	T	E,H
P. microps	Minor	A	N	T	H
Liopholidophis	Major	A	D	G	E,H,D,M
Pseudoxyrhopus	Hybrid	A	N	T	E,H,D
Heteroliodon	Hybrid	A	N	T	D,A
Liophidium	Minor	A	D	T	E,H,D,A

Major parent refers to species with greater genomic contribution to the hybrid lineage. Habitat is described as terrestrial (T), arboreal (Ar), or generalist (G), dentition is aglyphous (A) or opisthoglypohous (O), activity is diurnal (D) or nocturnal (N). Ecoregions refer to E = Evergreen rainforest; H = Central Highlands; D = Dry Deciduous; A = Arid Spiny Bush; M = Montane ericoid thicket.

well as sister species pairs (5/12), with an average parental divergence time of 2 million years (Figure 4, Supplementary Figure S3). Among the set of terminal taxa, there is no bias supporting reticulating lineages being younger on average (Supplementary Figure S4).

Additionally, the oldest reticulation we detected dates to the Miocene between 16.1 and 13.3 million years. We saw substantial discordance at the nodes joining these three clades (*Liopholidophis*, *Liophidium*, and *Pseudoxyrhopus+Heteroliodon*) and this reticulation was supported by all gene-tree methods when subsampling was performed. The site-specific method identified that 80% of triplets for this reticulation had a significant signal of hybridization. Often ancient hybrids are thought to exhibit extensive minor parent purging, with introgressed regions being relegated to small portions of the genome over time (Moran

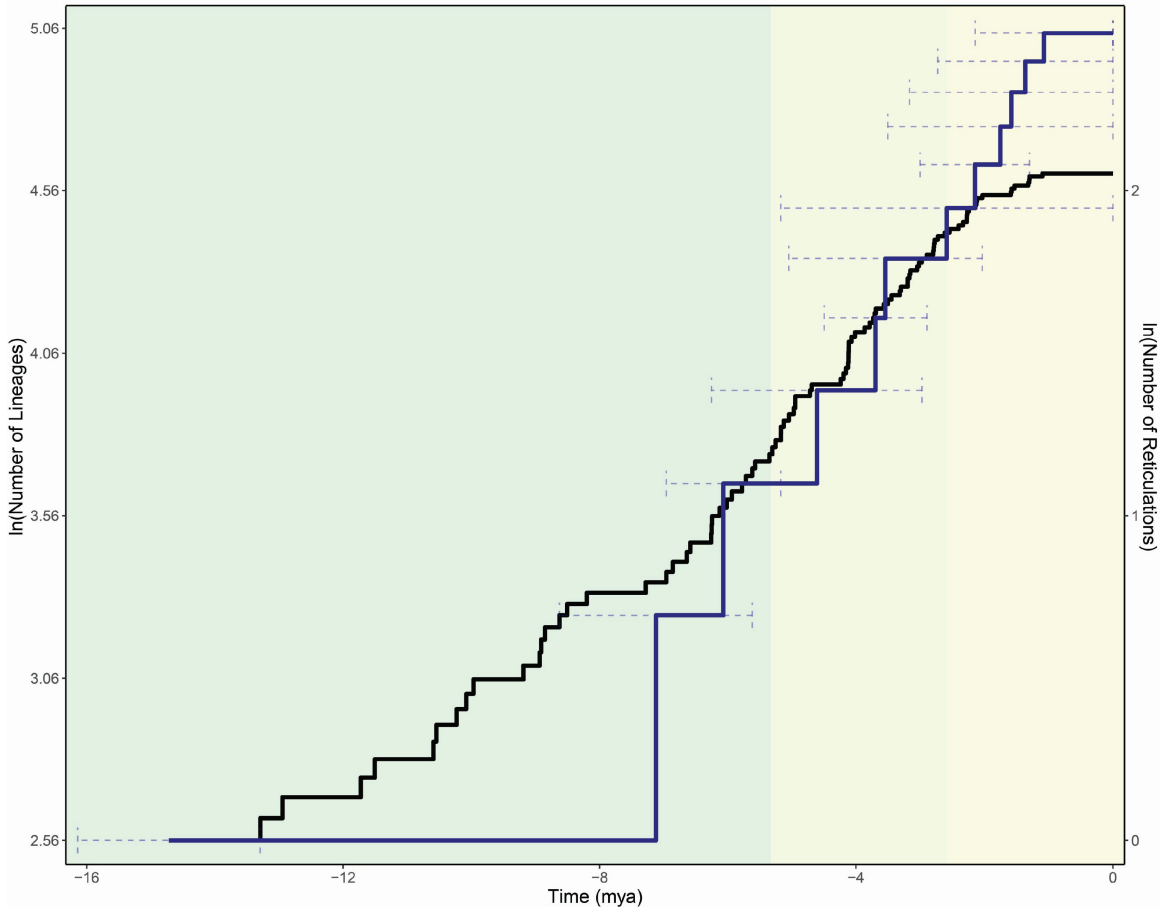

Figure 3. Reticulation through time plot. Blue line indicates reticulation accumulation through time, black is the lineage through time plot. Dashed horizontal lines are the error bars for timing of the reticulation. Time periods are depicted by the shaded regions: Miocene, Pliocene, Pleistocene (L to R).

et al., 2021). Clearly the signature of reticulation was not eroded in this case, with approximately 35% of genes derived from the minor parent, the ancestor to *Liophidium*. The reticulation propagated an 18-lineage radiation, which itself has two hybridization events.

As species accumulated in Madagascar throughout the Neogene, reticulations became more frequent, with six reticulations occurring in the Pleistocene alone. This is likely the result of more species accumulating on the island and the potentially small post-speciation dispersal for members of this group (supported in the biogeographical analysis presented here) causing species to interact and hybridize more frequently. The trend in overall speciation for the group is similar, as expected, with constant accumulation through time. However, in the Pleistocene, speciation rates decline (Burbrink et al., 2019; Figure 3). This may be attributed to interspecific competition, climatic, and environmental change. While these are factors that have been shown to affect bifurcating speciation (Storch & Okie, 2019), speciation by hybridization may be more robust to these processes.

As niche space is filled on a finite landscape like Madagascar it is expected that speciation rates should decrease if there are diversity-dependent limits (Pontarp & Wiens, 2017; Rabosky, 2013). However, hybrids exhibit greater genetic variation when compared to their parent taxa and may adapt to novel environments that remain unoccupied (Rieseberg et al., 2003). Hybrids can also have higher fitness than heterospecifics and have been shown to outcompete other species in several cases (Cutter & Gray, 2016). Madagascar also exhibited changing environmental conditions throughout the Pleistocene characterized by alternating glacial cycles (Burney et al., 2004). These periods may have affected speciation, owing to the decline in megafaunal assemblies in Madagascar (Crowley, 2010). Hybrids have been shown to perform well under climatic and environmental change; in suboscine bird species, the signal of introgression during the Pleistocene was highest in regions with more dynamic climates (Singhal et al., 2021). Additionally, neotropical cat hybrids were shown to be more amenable to climate change than their parental counterparts (Sartor et al., 2021). These factors may allow hybrid species to continue to accumulate even in the face of challenges that threaten other species (i.e., drastic environmental change and decreasing niche space). More studies at the genomic and ecological level are required to corroborate these hypotheses in this group.

Trends of ecology and biogeography

Out of the 17 genera examined here, twelve revealed species showing reticulation, and nine showed evidence of having hybrid lineages. We find reticulations among snakes with different ecologies (diurnal and nocturnal, arboreal and terrestrial, and aglyphous and opisthoglyphous) from all

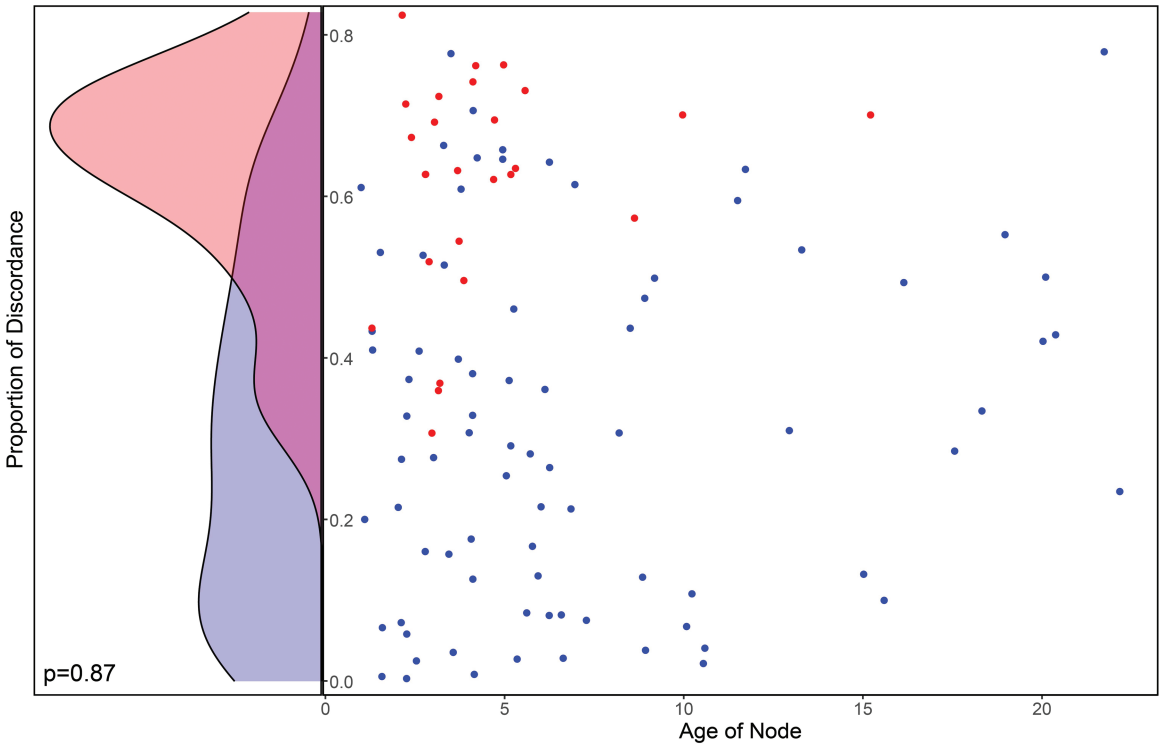

Figure 4. (R) Gene tree discordance of reticulating and non-reticulating nodes through time. Nodes involved in reticulations are in red, others are in blue. Overall discordance through time is poorly explained by regression with $r^2 = 0.005$, $p = 0.485$. (L) Density plot of proportional discordance for reticulating and non-reticulating nodes. Bootstrap p value shown.

ecoregions of Madagascar (Table 1). Interestingly, all hybridization events only include parent lineages sharing identical habitats and activity patterns with overlapping ecoregions of endemism. This suggests that species sharing similar habits are more likely to hybridize. For hybridization events with less than five terminal hybrid-parent lineages, the spatial location of hybridization logically follows the distribution of parent lineages; with the hybrid population located between them. Further, our hybrid populations also lie within 100km of at least one parent population (Supplementary Figure S6). This demonstrates that post-speciation dispersal has not eroded the spatial relationship between parents and hybrids for several million years. This is one of the first documented accounts of post-speciation dispersal limitations for herpetofauna in Madagascar.

In younger reticulations which have few descendants post-hybridization, we see a north to south distribution pattern for our reticulations (latitude sisters; Figure 5), rather than an east to west distribution pattern (longitude sisters). For example, *Langaha* and *Ithycyphus* are distributed across the island, but it is unclear within which habitat the ancestral parent resided. The eastern and western sides of the island exhibit opposite vegetation zones; along the east is a humid evergreen rainforest and to the west is a dry deciduous forest. The two biomes to the east and west are divided by a region of high altitude and different climates (Pearson & Raxworthy, 2009) having profound effects on regional reptile endemism (Raxworthy et al., 2003). The extreme environmental gradients between these biomes due to elevation/temperature and precipitation likely make interaction between the snake lineages uncommon. This would thus minimize the proportion of hybrid-parent regimes with this east-west distribution. On a north to south axis however, species that encounter each other are more likely to occur in the same ecoregion, which provides greater potential to produce successful hybrids (e.g., Gallego-Tévar et al., 2018; Otis et al., 2017).

From these findings, we identify that post-speciation hybridization is common. This can have important implications for downstream phylogenetic analyses. Unidentified reticulations will incorrectly infer branch lengths, speciation rate estimates, trait diversification, and other post-inference interpretations (Folk et al., 2018; Karimi et al., 2020). We noted that the trends in reticulation through time can also be shaped by biases in data that affect many kinds of evolution studies (Harmon et al., 2021). There are several factors that influence reticulation detection at both old and new timescales. Many network inference methods require information about one or both parents to accurately identify hybrids. Over time, the probability of extinction of both parents contributing to hybridization increases, therefore it is likely that hybrid detection is more difficult at deeper times. Additionally, many closely related taxa may show incomplete reproductive isolation, which will inflate the overall signature of recent reticulation relative to other species (Singhal & Bi, 2017). Here, many reticulation events involve younger and newly described species. How a species is delimited, depending on the concept used, may dictate prevalence of reticulation among younger taxa. It is possible that these biases affect interpretation on trends of hybrid species accumulation.

Future large-scale network analyses should be careful to evaluate reticulations in a multi-stepped, multi-method approach. Areas of discordance among our gene trees and discrepancies among different phylogenetic inference methods are associated with areas of reticulation (Figure 4). Given the computational limitations of network methods, phylogenetic trees currently must be split into multiple parts to

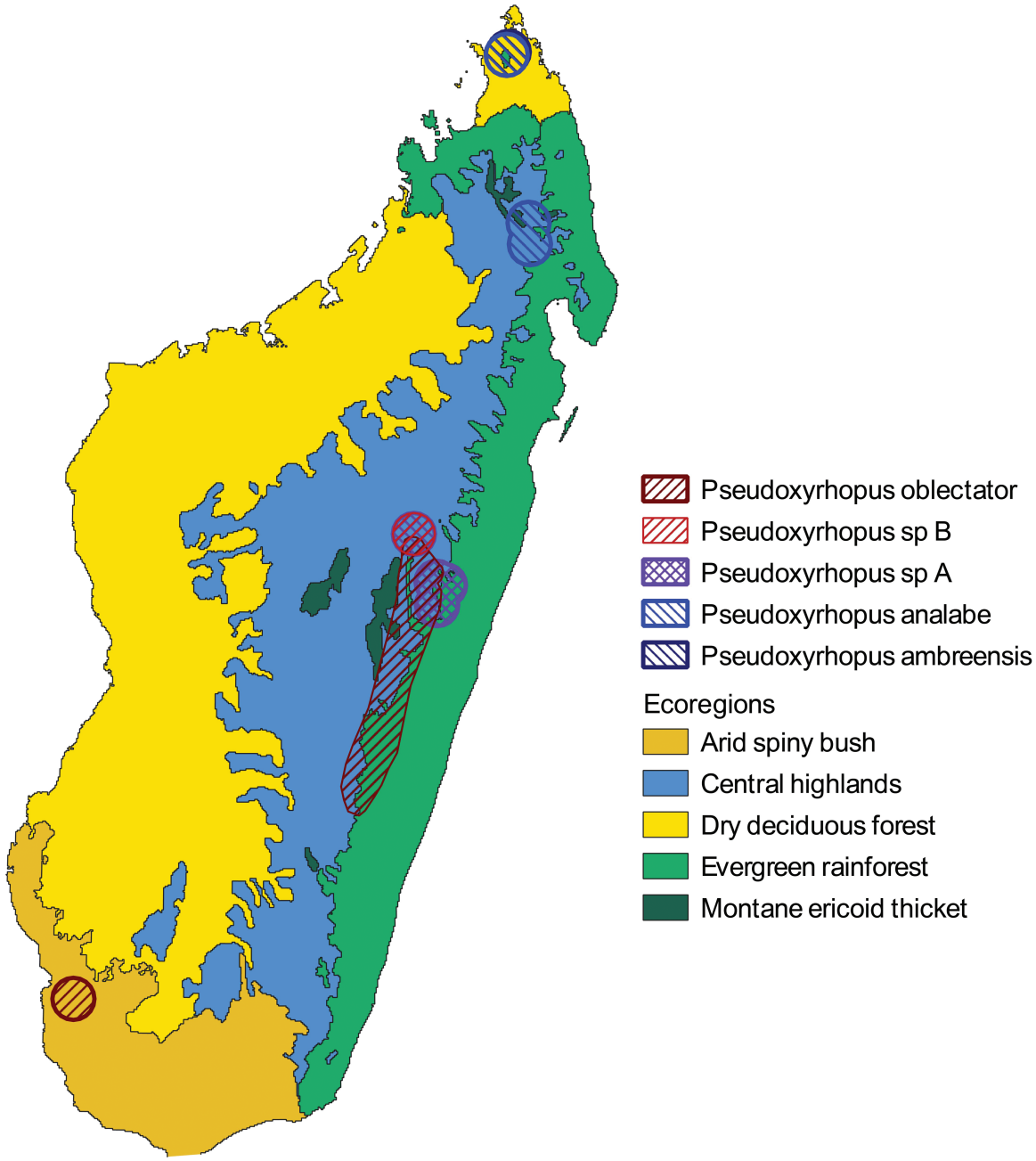

Figure 5. Distribution of *Pseudoxyrhopus sp A* and assumed parents. Purple denotes hybrid species range, red denotes major parent range, and blue denotes the minor parent range.

identify reticulate evolution, taking careful note of possible gene flow between the separated portions or excluded taxa. We were able to rule out reticulations between our subdivided groups with a test of monophyly, showing that most gene trees place these chosen clades in monophyletic groupings, and thus are concordant with the bifurcating species tree at this level. This was the case for all but one clade where we detected a deep time reticulation dating back to ~15 million years ago using subsampling efforts. We used several hybrid and network identification methods and statistical metrics to differentiate between probable networks. The pipeline for this analysis as well as the code is available on GitHub at dylandebaun/gemsnake reticulations.

Given advances in network detection methods and expanding explorations of deep time hybridization, we can better visualize the network of life as having many reticulations, with introgression substantially contributing to extant biodiversity. Continued research on the ecological and biogeographic mechanisms by which reticulation occurs will be crucial for understanding this process. In addition, with an increase in the availability of annotated whole genomes for non-model organisms, we can better understand the genetic mechanisms of introgression and the adaptations that drive ancient and recent reticulating parts of the genome across the network of life.

Supplementary material

Supplementary material is available online at *Evolution* (https://academic.oup.com/evolut/qpad011)

Data availability

Code is available on GitHub at dylandebaun/gemsnake-reticulations. Data is available on Dryad https://doi.org/10.5061/dryad.m0cfxpp7h

Author contributions

D.D. and F.T.B. conceived of the idea. D.D. conducted the analysis and wrote first draft of manuscript. F.T.B. and C.J.R. provided feedback. All authors edited the manuscript.

Conflict of interest: The authors declare no conflict of interest.

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