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RESEARCH ARTICLE

Testing molecular date estimates using an ecological and vicariant case study in treefrogs: The Geological-Ecological Molecular Calibration

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Abstract

Aim: To date a vicariant event through the mutualistic relationship of a hylid frog and its bromeliad host found across two isolated mountain ranges as part of an island–continental split and to use this information to calibrate a molecular dating analysis of hylids.

Location: South America.

Time Period: Pliocene.

Major Taxa Studied: Treefrogs, Arboranae.

Methods: Pliocene tectonic movements sank a portion of a mountain range that connected present-day Trinidad (island) and northern Venezuela, eventually isolating populations of the golden tree frog *Phytotriades auratus* and its host bromeliad *Glomeropitcairnia erectiflora* on both sides of a saltwater barrier. We estimated the submersion rate timing of the vicariant event, then employed ecological niche modelling to establish the distribution of the frog and its host to the Pliocene. We generated nucleotide sequence data for the hylid on each side of the marine barrier and proposed a biogeographic calibration for the split between populations. Using Bayesian phylogenetic analysis, we evaluated the impact of incorporating this biogeographic calibration on estimates of treefrog divergence times.

Results: A relatively continuous, high-elevation mountain range existed before the Pliocene and the opening of the Gulf of Paria (4.45 ± 0.85 Ma). *Phytotriades auratus* and *G. erectiflora* would have been distributed across the Paria and Northern Range mountains as a single population since the Pliocene. Divergence times among treefrogs (Arboranae) were older than those inferred using fossil calibrations alone,

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with mean age estimates for subfamilies and genera being ~2 and ~1.2 million years older, respectively.

Main Conclusions: Our study presents a biogeographic calibration based on a vicariant model that connects the evolution of the populations of a hylid through geology and ecological data. The implementation of this date as a molecular clock calibration reduces the uncertainty in date estimates for the shallower nodes in the phylogeny of Arboranae.

KEYWORDS
divergence, genetic divergence, hylid, molecular dating, tectonics, vicariance

1 | INTRODUCTION

Knowing the timing of species divergences is key to understanding the underlying evolutionary processes. Inferences from genetic data can be made using molecular clock methods, but these need calibration based on external information about specific events. For this purpose, fossil evidence or geological events are most often used (for recent reviews see Landis, 2020; Nguyen & Ho, 2020). When geological events are assumed to form a barrier to gene flow (i.e., vicariance events), their dates can be assigned to corresponding nodes in the phylogeny (Ho et al., 2015). However, clades can be older than the inferred geographic isolation (Jones et al., 2009, 2013; Marko, 2002; Parent et al., 2008), even though some authors argue that evolutionary divergence times must match the timing of geological events under a strict vicariance scenario (e.g., Crisp et al., 2011). Furthermore, mechanisms such as transoceanic dispersal may postdate barrier formation (Kodandaramaiah, 2011). Such events have been suggested for low-vagile species such as amphibians (Vences et al., 2003) and fossorial reptiles (Vidal et al., 2008). Therefore, the accuracy and precision of clock calibrations based on geologic events can potentially be improved by incorporating environmental information.

An example of lack of accuracy and precision in geology-based events is the widely used geological calibration based on the formation of the Isthmus of Panama. This land bridge facilitated the Great Biotic Interchange between North and South America about 2.8 Ma (Leigh et al., 2014). However, recent evidence suggests that land bridges were already present in the Middle Miocene 16 to 11.6 Ma and even up to 6 Ma (Bacon et al., 2015; Montes et al., 2015), so faunal interchange is likely to have occurred more than once. Similarly, many studies of European taxa have used molecular clocks calibrated by the Messinian Salinity Crisis evaporitic phase and the post-evaporitic phase or Lago Mare phase between 5.61 and 5.33 Ma (Stock et al., 2006). However, the Messinian Salinity Crisis created an imprecise transitional range of conditions that were not equally favourable to all species (Akin et al., 2010). Therefore, including ecological constraints can improve the precision of calibrations based on geological models.

Geological barriers impose different constraints on taxa depending on their biological characteristics (e.g., dispersal capability, colonization success, and ecological niche specialization). Hylid frogs, for example, are often highly specialized to their ecosystems, where they rely on moisture to prevent their naked skin from drying out, which represents a strong physiological constraint to dispersal through a marine barrier. Thus, there is a need to address these ecological and physiological issues in each study independently, but pertinent information is often unavailable for vicariant events. Calibrations should also account for the prolonged nature of geological events, as sustained barriers to gene flow often form gradually (Upchurch, 2008). For this reason, calibration priors with soft bounds (exponential/lognormal) rather than hard bounds (fixed/uniform) are typically used in Bayesian phylogenetic analyses.

Here we propose a vicariant model based on a mutualistic relationship between a hylid frog (the golden tree frog, *Phytotriades auratus*) and its host (the giant bromeliad *Glomeropitcairnia erectiflora*). The example adheres to the strict association of ecological (an adaptation to life in a bromeliad niche and frog larval development in phytotelmata) and abiotic (altitude and cloud forest habitat) conditions linked to empirical geological data to estimate the time of divergence of the populations/species on each side of the barrier. Until recently, this frog was only known from two mountain summits on the Caribbean island of Trinidad (Republic of Trinidad and Tobago), where its populations were isolated and restricted to low-altitude cloud forests. Analyses of environmental DNA confirmed its presence in other small patches of cloud forest on additional peaks on the island (Brozio et al., 2017; Torresdal et al., 2017). The placement of this species in a monotypic genus (Jowers et al., 2008) distinguishes it from the one it was previously assigned to, *Phyllodytes*, which is now endemic to southeastern Brazil. Recent work has established the presence of a new population or subspecies in Venezuela's Peninsula de Paria, hosting a mountain chain that was connected to Trinidad's Northern Range until the Early Pliocene (Rivas & De Freitas, 2015). Hence, a 7 km stretch of open water in the Gulf of Paria now separates the Trinidadian and Venezuelan populations (Figure 1).

Mainland and island populations of *P. auratus* share the same close association with the giant bromeliad *G. erectiflora*, which is also endemic to Northeastern Venezuela and Trinidad. All frog observations have been on or within this plant in cloud forests above 700 m in elevation. Presumably, the golden tree frog's requirement

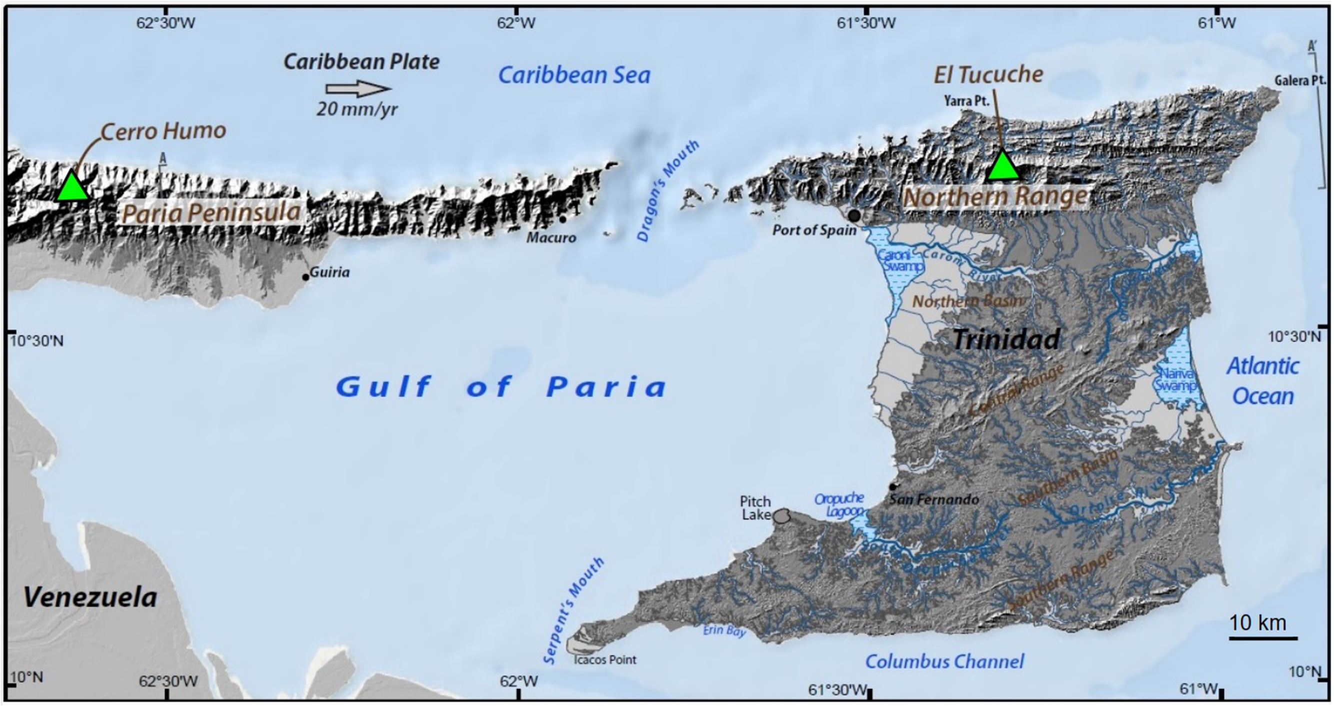

FIGURE 1 Index map showing modern topography of the island of Trinidad (Republic of Trinidad and Tobago) and the Paria Peninsula of Northeastern Venezuela. Green triangles are sampling localities of *Phytotriades auratus* individuals included in the phylogenetic analyses.

for bromeliads containing large volumes of water relates to its reproductive mode, which involves laying eggs in the water and tadpoles feeding on the algae growing on the plant tank walls. In fact, adults of *Phytotriades* have an extremely dorso-ventrally compressed body, which is likely to be an adaptation for living between bromeliad leaves (Clarke et al., 1995).

Given their present strict association and restriction to low-altitude cloud forests (700–1250 m a.s.l.), the golden tree frog and giant bromeliad are likely to have been constrained to their present-day distribution in the recent past (Pleistocene–Holocene), although much older Pliocene distributions are unknown. This is supported by the lack of suitable habitat and geographical barriers beyond the limits of their distribution (southern areas of Paria Peninsula and north of Trinidad). Genetic isolation and high genetic divergence of *P. auratus* from other hylid frogs and of *Glomeropitcairnia* from other bromeliads are probable considering the isolation at these localities. Knowledge of the range sinking rate can be linked to the time both populations were last in contact, which is estimated to have been when mountain summits sank to circa 700 m above sea level, thus yielding unsuitable humidity conditions for both the bromeliad and the hylid.

In this study, we aim to improve the precision of the vicariance date of this host-guest system using environmental, ecological, and geological information and to evaluate the impact of our dating approach on the Hylidae phylogeny. More specifically, we employ statistical methods on empirical geological data to estimate the Gulf of Paria's sinking rate. Ecological niche modelling of both the bromeliad and its host frog, with projections to the past distributions, correlate with the sinking of the mountain range to give a precise estimate of the time of separation of both populations. Using this timing to inform an additional calibration reduces the uncertainty in date estimates for the shallower nodes in the phylogeny of hylid treefrogs. Our work outlines the relevance of the Geological-Ecological Molecular Calibration (GEMC) concept and applies it to a molecular dating analysis of amphibian clades.

2 | MATERIALS AND METHODS

2.1 | Understanding the geology and geomorphology of the coastal mountains and region

Both *P. auratus* and *G. erectiflora* are endemic to the coastal mountains of Venezuela's Paria Peninsula and Trinidad's Northern Range (henceforth referred to as the Eastern Coastal Range in Venezuela and Northern Range in Trinidad) (Jowers et al., 2008; Rivas & De Freitas, 2015). These moderate elevations (<1250 m) consist of a narrow E–W trending belt of metamorphic rock along the southern edge of the broader Caribbean mountains that rims NE South America (Figure 1) (Rivas et al., 2021). The metamorphic geology and initiation of high topography in the mountain range occurred primarily in the Miocene, when oblique collision of the Caribbean plate with South America caused a crustal welt to develop. Miocene crustal shortening and thickening drove rock exhumation and surface uplift of the coastal metamorphic mountain range (Arkle et al., 2021; Cruz et al., 2007; Frey et al., 1988; Weber et al., 2001). The protolith or precursor rocks of the metamorphic belt date back to the Mesozoic (Algar et al., 1998; Pindell et al., 1998). Trinidad's western islands are interpreted as submerged relicts of a former continuous high-elevation range with low-elevation peaks now standing just above sea level. Few studies address Paria's geomorphology (Arkle, Owen, Weber, Caffee, et al., 2017; Arkle, Owen, & Weber, 2017; Cruz et al., 2007; Ritter & Weber, 2007), yet a similar west-to-east transition of topography is observed, with elevations increasing westward in Paria. This mountainous coastal landscape is interpreted to reflect symmetric and active sinking into the Gulf of Paria pull-apart basin (Arkle, Owen, & Weber, 2017; Arkle, Owen, Weber, Caffee, et al., 2017; Ritter & Weber, 2007).

The Gulf of Paria is a shallow brackish waterway that connects the major seaways around Trinidad. Tectonically, it is a pull-apart basin that links the right-lateral El Pilar strike-slip or transform fault in Venezuela to the Warm Springs–Central Range transform fault in Trinidad. The two transform faults take up the bulk of the current ~20 mm/year, eastward, relative Caribbean–South American plate motion (Weber et al., 2020). The right-stepover of the faults forms a ~40 km wide zone of local extension, which accommodates stretching, and subsidence of the crust in the Gulf of Paria. The geology and sedimentary fill in the Gulf of Paria has been fairly well characterized by extensive well data and seismic profiling related to oil exploration and extraction, which yield evidence that opening in the Gulf began in the Early Pliocene 4.45 ± 0.85 Ma (Figure 2) (Babb & Mann, 1999; Flinch et al., 1999).

2.2 | Geology-biology connections and model

Initial isolation of Cerro Humo and El Tucuche-Cerro del Aripo subpopulations began once westward-sinking (Trinidad) and eastward-sinking (Paria) of mountain top elevations subsided into the Gulf below the ~700 m habitat range of *P. auratus* (Figures 2 and 3). To determine subsidence and the timing of physical isolation, we model toward a relatively continuous high-elevation (600–1000 m) mountain range that existed prior to the Pliocene and the opening of the Gulf of Paria. The structurally deepest part of the Gulf of Paria, where the sediment pile is thickest and the basin floor is lowest, was located using the high-quality cross-sections of Flinch et al. (1999) (i.e., see their cross-section II).

We measured a compacted sedimentary rock thickness of post-4.45 Ma age as Pliocene—1.2 s and Pleistocene—1.4 s, in two-way travel time. We then divided each of these values by 2 to get one-way travel time. We next multiplied one-way travel time by rock velocity, which is between 1888 and 2000 m/s as determined from oil and gas production wells off the north coast of Trinidad, which has geology very similar to that in the Gulf (De Verteuil, 2005, personal communication). We obtain a total post-4.45 Ma rock thickness of between 2455 and 2600 m. Next, following Bond and Kominz (1984), we de-compacted these rock thicknesses (assuming 85% mudstone with a 45% porosity change and 15% sandstone with a 10% porosity change) to get original sediment thickness of between 3433 and 3633 m. We hence divided this number by time (4.45 Ma) to obtain a sedimentation rate in the deepest part of the basin of between 0.77 and 0.82 mm/year. Assuming that the Gulf of Paria basin was filled to the top as it subsided, these sedimentation rates are reasonable proxies of subsidence rates, the magnitude of which is also supported by empirically measured erosion (Arkle, Owen, Weber, Caffee, et al., 2017) and exhumation (Arkle et al., 2021) rates (0.5–1.5 mm/year) since the Mio-Pliocene. In addition, the modern Orinoco River has continually transported large sediment volumes <200 km away from the high Andes to Trinidad and eastern Venezuela since the Late Miocene (Babb & Mann, 1999).

2.3 | Species occurrences and environmental data

To investigate the historical distribution range of the giant epiphytic bromeliad in Venezuela and Trinidad, we used ecological niche modelling (Elith & Leathwick, 2009; Sillero et al., 2021) based on the interactions between species distribution and related climate factors (Figure S1). For this purpose, we used 28 GPS species locations (ranged from 700 to 1056 m a.s.l.) from local surveys from two data sources (Tables S1 and S2). Bromeliad presence has never been reported at altitudes lower than 700 m in the literature or throughout our own past surveys in Paria Peninsula, Margarita, and Trinidad (Clark et al., 1995; Jowers et al., 2008; Rivas & De Freitas, 2015). We are aware that physiological tolerance of both the frogs or the bromeliad cannot be assumed to have been identical throughout their past to present conditions, but we assume a similar past altitude range to those at present as the better proxy for our models. In addition, the abundance of elevations above 700 m suggests larger populations of both frogs and bromeliads distributed at higher (>850 m) rather than lower altitudes (<850 m). To avoid spatial autocorrelation, we used the ‘spThin’ R package (Aiello-Lammens et al., 2015) to reduce clusters in the species occurrence records (Sillero et al., 2021).

All occurrences were re-projected to match with the spatial resolution of predictors, giving them a 20 presence-only species data set (centroids of a 1 km^2 grid). As climatic conditions exert a strong control over the species geographic distribution (Zotz, 2016), 19 current (bio-) climate variables (BIO1 to BIO19, Table S3) were downloaded from the CHELSA database v.2.1, corresponding to the highest resolution (30 s resolution, ~1 km^2 grid). We also considered topographical variables since geomorphology has an important impact on giant bromeliad survival (Zotz, 2016). The altitude was derived from the GTOPO30 Global Elevation Data Earth Resources Observation and Science Center/U.S. Geological Survey/U.S. Department of the Interior (1997) with a 30 s resolution (~1 km^2 grid) (Research Data Archive at the National Center for Atmospheric Research). We then computed slope and aspect variables through spatial analysis tools in Quantum GIS 3 (Quantum GIS Development Team, 2017).

To avoid including highly correlated variables in model fitting, we conducted a multicollinearity analysis through Pearson pairwise correlations and the variance inflation factor (VIF). Based on this approach, we then retained only four independent predictors with Pearson correlation coefficients <0.7 and VIF <2 (Figure S2): BIO11 (mean temperature of coldest quarter), BIO15 (precipitation

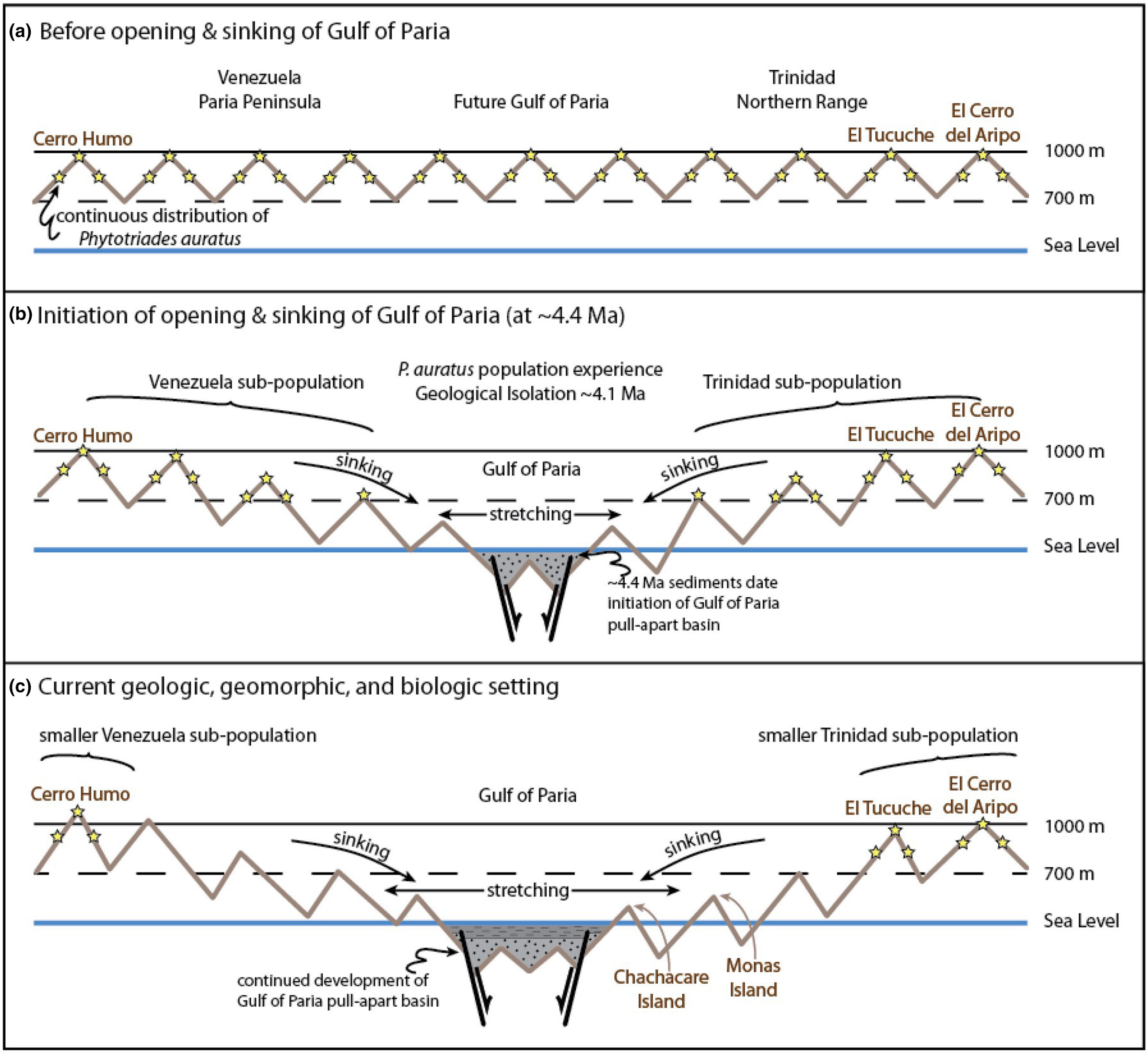

FIGURE 2 Geologic-geomorphic-biological model showing the evolution of topographic elevations and *Phytotriades auratus* biological isolation as a time-series with panel (a) before opening and sinking of the Gulf of Paria (pre 4.45 ± 0.85 Ma), (b) at the start of opening sinking 4.45 ± 0.85 Ma, and (c) today showing the current geologic, geomorphic, and biologic setting.

seasonality [coefficient of variation]), BIO18 (precipitation of warmest quarter), and slope. These attributes were then used as the best predictor variables for the species presence in subsequent model fitting. Once we selected the current climate attributes, we obtained the same variables for the past based on the PaleoClim database (Karger et al., 2017) for the following time periods: (1) mid-Holocene, Northgrippian (~6000 years; MH) and (2) Last Interglacial (~120,000–140,000 years; LIG) within the Pleistocene; (3) mid-Pliocene warm period (3.2×10^6 years; mPWP); and (4) Marine Isotope Stage M2 (ca. 3.3×10^6 years) within the Pliocene. Thus, a niche model for the present-day distribution of the giant bromeliad was created for Venezuela and Trinidad, and it was further projected in the past (MH, LIG, mPWP, and M2) to the highest resolution available (2.5 arc-min, ~5 km^2 grid).

2.4 | Model fitting

To obtain spatial projections of potential habitat for bromeliad species, we calibrated models using an ensemble forecasting approach based on the nine modelling techniques implemented in the ‘biomod2’ R package (Thuiller, 2014): (1) artificial neural networks (ANN); (2) classification tree analysis (CTA); (3) flexible discriminant analysis (FDA); (4) generalized additive model (GAM); (5)

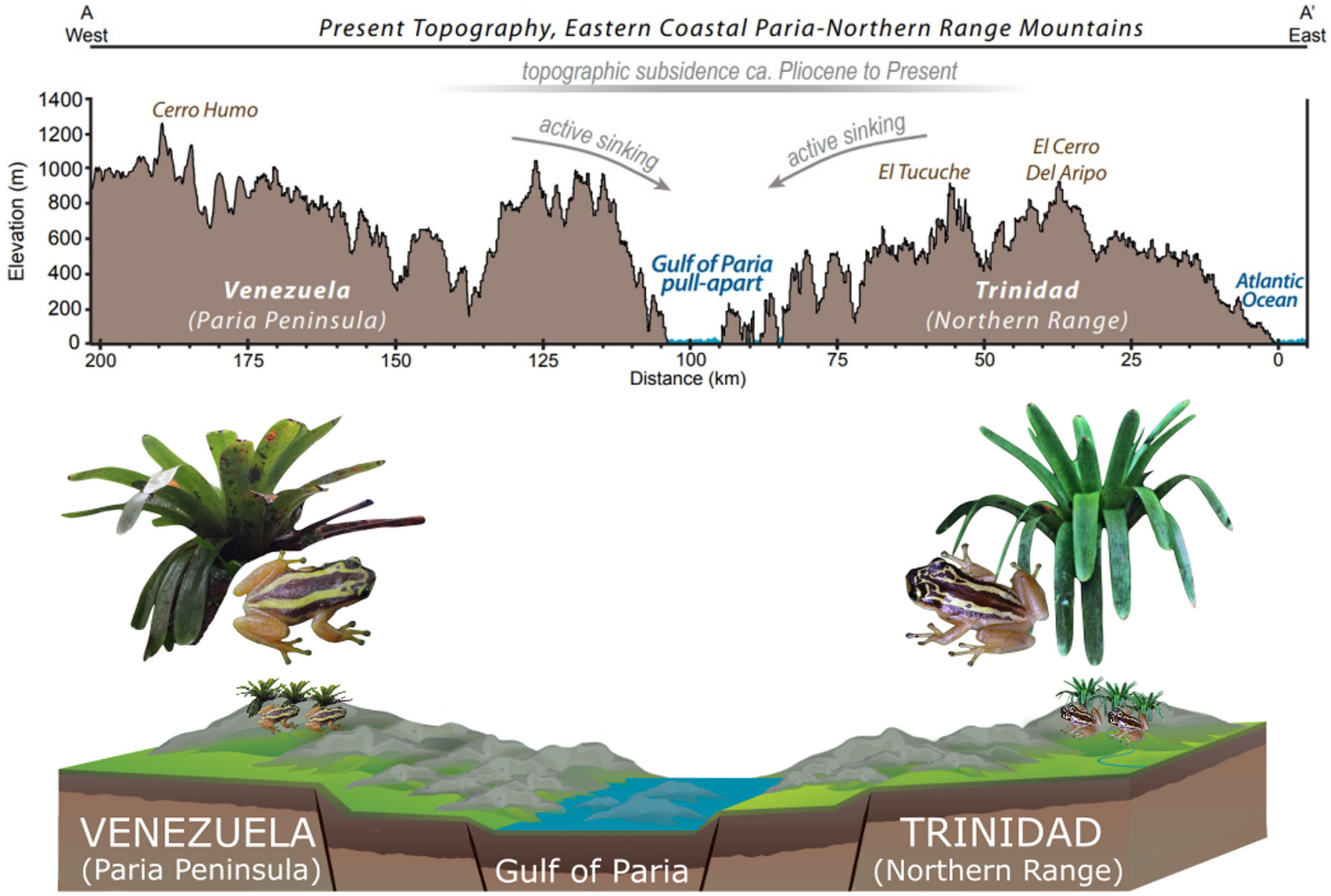

FIGURE 3 Top: maximum topographic profiles. *Phytotraides auratus* subpopulations are found today on mountain peaks (Trinidad: El Cerro del Aripo, El Tucuche; Venezuela: Cerro Humo) at elevations of ~1000 m. The Gulf of Paria represents a sunk region that formed a geographic barrier and isolated Venezuelan and Trinidadian subpopulations starting from 4.1 ± 0.85 Ma. Bottom: graphic representation of Paria Peninsula and Trinidad's Northern Range sinking and flooding of the Gulf of Paria. Picture of the bromeliad and frog in Paria (credit to Tito R. Barros), and the pictures of the ones in Trinidad (credit to John C. Murphy).

generalized boosted models (GBM); (6) generalized linear model (GLM); (7) multivariate adaptive regression splines (MARS); (8) maximum entropy using Phillip's Maxent software (MAXENT); and (9) random forests (RF). Default parameters were used for all modelling techniques, with the exception of the smoothing degree term in GAM algorithm which was set to $k = 4$, and the number of boosting trees in GBM (n.trees = 2000) to prevent over-fitting (Guisan et al., 2002) (Figure S1).

We also generated 10 sets of randomly distributed pseudo-absences (PAs) in the model calibration. PAs were generated by assigning unoccupied grid cells with the following constraints: (1) generating the same number of PAs as of presences to avoid potential bias caused by different levels of prevalence in the presence/absence data sets (as recommended by Barbet-Massin et al., 2012; Manel et al., 2001) and (2) defining a minimum distance between PAs, corresponding with the grain size (5 km), and without overlapping with presences (Wisz & Guisan, 2009), to avoid spatial autocorrelation and to cover the different ecological conditions in the study area. Each model was fitted using 70% of the data and tested using the remaining 30%.

We employed hold-out cross-validation to evaluate the models with 10 evaluation rounds for each set of PAs. The predictive performance and discrimination ability of individual models were evaluated using two metrics (Baasch et al., 2010): (i) the area under receiver-operating-characteristic curve (AUC) and (ii) and the true skill statistic (TSS). The AUC ranges between 0 and 1 (models with AUC ≥0.7 were considered good), while TSS ranges from 0 or less than 1 (models with TSS ≥0.4 were considered good). To deal with uncertainty in our models coming from the single-algorithm techniques, we built ensemble (consensus) models among those satisfying the conditions AUC ≥0.7, and TSS ≥0.4. We used the weighted mean of all the partial projections (Marmion et al., 2009), a consensus method that considers the weights proportional to the selected evaluation scores (i.e., the higher the TSS of the model, the greater its importance in the ensemble modelling; Konowalik & Nosol, 2021). Finally, we performed binary transformations of the predicted probabilities into suitable/unsuitable areas predicted by the ensemble models based on the TSS-optimized thresholds available in the 'biomod2' R package (Thuiller, 2014) (Figure S1).

2.5 | Molecular phylogenetic analyses

DNA was extracted from tissue samples of four individuals of *P. auratus* (three from Paria Peninsula, locality Cerro Humo, and one from Trinidad, locality El Tucuche) (Table S4) using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and following manufacturer's instructions. We amplified the mitochondrial *12S* and *16S* rRNA genes along with tRNAs, partial sequences of mitochondrial cytochrome *c* oxidase subunit I (*COI*), cytochrome *b* (*CYTB*), and NADH dehydrogenase subunit 1 (*ND1*) and tRNA, as well as nuclear proopiomelanocortin (*POMC*) and recombination-activating protein 1 gene (*RAG-1*) (Supporting Information; Table S5; see also "Data Availability Statement" section). These markers are informative in interspecific and intraspecific studies of frogs (Faivovich et al., 2005). The new sequences were aligned with those of Duellman et al. (2016) (which also included a *P. auratus* from El Tucuche, Trinidad), using MAFFT (Katoh et al., 2002). The complete DNA matrix included 17,017 aligned base pairs from 511 taxa.

Phylogenetic analyses were performed using Bayesian and maximum-likelihood methods. We identified the best-fitting partitioning scheme and substitution models using the Bayesian information criterion in PartitionFinder v.2 (Lanfear et al., 2017) (Table S6). Maximum-likelihood analysis was performed using RAxML v.7.0.4 (Silvestro & Michalak, 2012), with node support assessed using 1000 bootstrap replicates.

We inferred the phylogeny, divergence times, and substitution rates using the Bayesian approach implemented in BEAST v.2.6 (Bouckaert et al., 2014) with the bModelTest package (Bouckaert & Drummond, 2017). Sequences were concatenated, and the data set was partitioned. We used an uncorrelated exponential relaxed clock while allowing a relative rate parameter for each partition (see XML files) (Drummond et al., 2006). To reduce computational load, we fixed the tree topology to that estimated in our ML analysis. We sampled from two independent MCMC chains of 125 million steps each, sampling every 10,000 steps. After removing 10% of the samples as burn-in, we checked for convergence between the two runs and sufficient sampling using Tracer v.1.7 (Rambaut et al., 2018). We combined the samples from the two chains. All analyses were performed on the Niagara Supercomputer hosted by the SciNet Consortium at the University of Toronto (Ponce et al., 2019).

We ran two Bayesian analyses that differed in the number of calibration points. First, we replicated the calibrations used by Duellman et al. (2016) by specifying uniform priors on the ages of three nodes: (1) minimum divergence time of 16 Ma between Hylinae and Acridinae, based on *Hyla* remains from the Miocene, (here setting an arbitrarily high value for the upper bound was not sufficiently informative, so we instead used an exponential prior to provide a soft upper bound, with an offset lower bound of 16 Ma); (2) divergence time of 35–70 Ma between Phyllomedusidae and Pelodryadidae based on the timeframe when Australian pelodryadid arboranans (represented by *Litoria*) could disperse from South America through Antarctica; and (3) maximum age of 37.2 Ma for the Caribbean genus *Osteopilus*, the date when the Greater and Lesser Antilles became permanently subaerial, based on geologic evidence. In our second dating analysis, we added a uniform calibration prior for *Phytotriades* with bounds of 3.25 and 4.95 Ma. These dates are based on the geological data described in this study (see Section 3).

To compare divergence times between the two inferred time-trees, we imported the maximum-clade-credibility trees and 1000 randomly selected trees from the posterior sample into R. We then used the *branching times* function to extract the divergence times for each tree and multiple subclades at the family level. Finally, we produced lineages-through-time plots using the *rBt* (Sánchez-Ramírez, 2018) and *ggplot2* R packages.

3 | RESULTS

3.1 | Geological estimate: Venezuelan–Trinidadian *P. auratus* sub-population isolation age

Our geologic model connects the evolution of the two subpopulations of *P. auratus* by a relatively continuous high-elevation (600–1000 m) mountain range that existed prior to the Pliocene and the opening of the Gulf of Paria. During the Miocene, the crust along the South American–Caribbean margin was contracted and tectonically thickened (Algar et al., 1998; Speed et al., 1991; Weber et al., 2001). Crustal shortening during the Miocene was the primary stage of mountain uplift and building of high-elevation topography, with rapid rates (up to ~1.5 mm/year) and large magnitudes (over ~7 km) of rock exhumation (Arkle et al., 2021). *Phytotriades auratus*, confined today to elevations between 700 and 1250 m, would have been distributed across Paria and Northern Range mountains as a single population pre-Pliocene (Figure 2).

In the Pliocene, tectonic interactions inverted to extension, causing a net lowering of the mountain belt by both east–west lengthening and vertical subsidence (Arkle et al., 2021; Arkle, Owen, Weber, Caffee, et al., 2017; Babb & Mann, 1999; Ritter & Weber, 2007). Pliocene vertical collapse of the crust progressed (and still does today) eastward and westward from roughly the centre of the Gulf of Paria (Babb & Mann, 1999; Flinch et al., 1999; Ritter & Weber, 2007; Weber, 2005). As the coastal mountains sank, the once continuous and high-elevation habitat that supported *P. auratus* was reduced progressively eastward and westward from the centre of the Gulf below their threshold elevations. Using geology and the methods outlined above, we calculate that initial isolation of the *P. auratus* subpopulations occurred at 4.1 ± 0.85 Ma.

Continual subsidence is evident today in the Dragon's Mouth Passage that separates Trinidad from Venezuela: there, the lowest bathymetric elevations (~200 m) are near the centre of the passage and what were likely once high catchment divides have formed isolated islands (e.g., Chacachacare) and farther drainage divides are at or near sea level (e.g., to the west in Paria, the Mejillones Cove, and to the east in Trinidad, Tucker Valley). Peaks currently reaching the ~700 m *P. auratus* threshold elevations are relicts of the once higher topography. In addition to sinking, today's peak elevations are also affected by erosion (modern rates ~50 m/Ma) that also wears down the topography (Arkle, Owen, Weber, Caffee, et al., 2017). The overall subsidence in the region of study is also likely enhanced by local small-scale faults, such as the fault block system that has dropped down the Mejillones Cove in eastern Paria.

3.2 | Ecological niche models: Variables and contemporary and historical bioclimatic niche modelling

All models were statistically more robust than the random one ($AUC_{null}=0.65$), with an average AUC of 0.98 ± 0.005 and range of 0.78–1. The highest relative contribution of each variable ranged from mean temperature of coldest quarter (BIO11: 40.95%), precipitation seasonality (BIO15: 20.59%), Slope (19.49%), and precipitation of warmest quarter (BIO18: 15.81%).

The current suitable habitat for bromeliad *G. erectiflora* in the study area reached 23.57% (Figure 4a; Table S7). The MH (~6000 years) past output projection revealed that more than 50% of the total area was represented by 50%–100% probability for the species occurrence (Figure S3b), reaching 38.46% of suitable area (Figure 4b). The median-high likelihood of the species presence was found in a large portion in the middle of the study area and the Northwestern region of Venezuela, and mainly in Trinidad's north region (Figure 4b). In both cases, the highest suitable area values matched with the present-day species distribution along Paria Mountain range (north Venezuela), and Northern Range in Trinidad, where more than 80% of the suitable habitat ranged from 850 to 1000 m a.s.l. (Mount Tucuche, 936 m, and Cerro del Aripo, 940 m). A significant suitable area was also projected along an altitudinal gradient (600–850 m a.s.l.) to both Isla Margarita and Tobago.

The LIG scenario (~120,000–140,000 years) had similar suitable area for bromeliads as MH (37.79%), and more than 70% of the total area had a probability of presence over 60%. As in the case of the MH, the areas with highest likelihood of bromeliad presence matched with the present-day distribution of species (Figure 4c), as also in Trinidad, and following an altitudinal gradient above 700–850 m in all cases.

Both mPWP and M2 Pliocene scenarios showed similar patterns, reaching 52.74% and 40.96% of suitable habitat, respectively (Figure 4d,e; Table S7). The areas with highest likelihood of bromeliad presence (>60% of probability) appeared along the Paria Range, Northwestern and Margarita Island in Venezuela, the Northern Range in Trinidad, and almost full area in Tobago (Figure S3b; Table S7).

3.3 | Differences between calibration timings and mean rates

Divergence times inferred using the combined fossil and GEMC calibrations were older than those inferred using the fossil calibrations alone (Figure 5). The two trees put the divergence between the Venezuelan and Trinidadian populations at 3.46 and 0.94 Ma, respectively. When the GEMC calibration was included, average age estimates for subfamilies (excluding *Phytotriades*) were 2 million years older (8.1%; Figure 6; Table S8), while genera were 1.2 million years older (8.5%; Table S9; Figure S4). Nevertheless, although the posterior medians are slightly different, the 95% credibility intervals show considerable overlap, suggesting that the addition of the GEMC calibration had no substantial effect on the estimated divergence times.

Inferred substitution rates were lower when the GEMC calibration was included, with the mean rate across all genetic markers being 7.61% (0.0133 vs. 0.0144 substitutions/site/Ma) lower in the subfamilies. This average excludes the subfamily Lophyohylinae as it includes *Phytotriades* (10% faster) (Table S10). Similarly, we inferred substitution rates that were 7.89% (0.0134 vs. 0.0146 substitutions/site/Ma) lower in genera, again excluding *Phytotriades* (22.02%, mean 0.0131 vs. 0.0168 from this mean estimate) (Table S11). Estimates of substitution rates of individual markers are given in Table S12.

4 | DISCUSSION

Our study has shown that an ecological vicariance event can be used to generate an informative calibration for molecular clocks. We consider that the reliability of the information used for the biogeographic calibration estimated here is rarely found among vertebrates, as, to the best of our knowledge, there are no other similar cases of such intrinsically linked causal factors leading to multiple strict associations. Our calibration is based on the strict association of *P. auratus* to high-elevation cloud-forest giant bromeliads *G. erectiflora* as its habitat, as well as the dependency of tadpoles for feeding on its phytotelmata microalgae. This calibration is supported by ecological data of a connective corridor of this habitat in the past and geological evidence and estimated dates of the range of submergence.

Traditionally, biogeographic processes that promote allopatric speciation are vicariance, geodispersal, and biological dispersal, the last of which is common throughout South American islands (Barbo et al., 2022; Cicchi et al., 2007). However, the complexity and habitat–host relationship of this particular model does not accommodate the time lines suggested by other studies that have addressed colonization patterns in the island of Trinidad. In particular, Trinidad's only endemic frog, the dendrobatid stream frog (*Mannophryne trinitatis*), split from its sister species from Paria Peninsula in the Late Miocene (ca. 7 million years; Jowers et al., 2011). This timing was attributed to vicariance based on the available geological data (Audemard & Audemard, 2002; Moretti, Delos, Letouzey, & Calvo, 2007; Moretti, Delos, Letouzey, Otero, et al., 2007; Persad, 2009; Tyson et al., 1991; Vierbuchen, 1984). Results from the present study imply that a ~7 Ma split betwen Paria–Trinidad *Mannophryne* would predate when Trinidad broke off completely from the Paria Peninsula, suggesting a nonvicariant event. Other species from this region that have colonized Trinidad through land bridge connections during

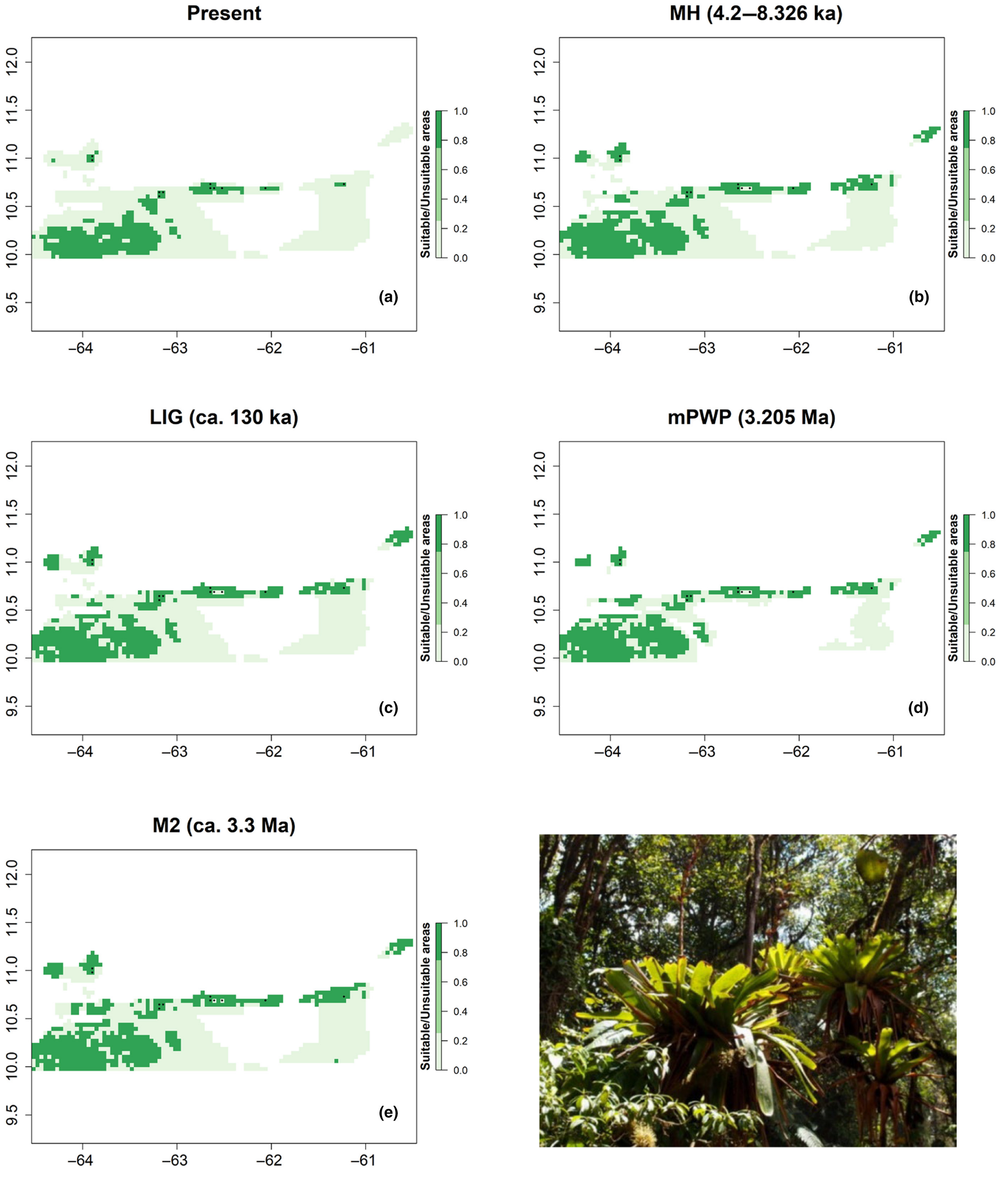

FIGURE 4 Suitable and unsuitable areas for *Glomeropitcairnia erectiflora* based on ensemble models for each climatic period (a–e). Colour ramp ranged from worse predictions (light green) to better predicted conditions (dark green). Black dots show the species presence locations at ~1 km^2 grid. See text for details. Bottom-right figure are *G. erectiflora* at El Tucuche (credits to Mike Rutherford).

glacial periods (Jowers, Othman, et al., 2021) are either present in nearby Venezuela (Murphy et al., 2016, 2017), have high genetic similarity to the mainland counterparts (Jowers et al., 2015, 2019), or have used connective land bridge corridors even to the oceanic island of Tobago (Jowers et al., 2015; Jowers, Othman, et al., 2021; Jowers, Schargel, et al., 2021). For example, the habitat-generalist

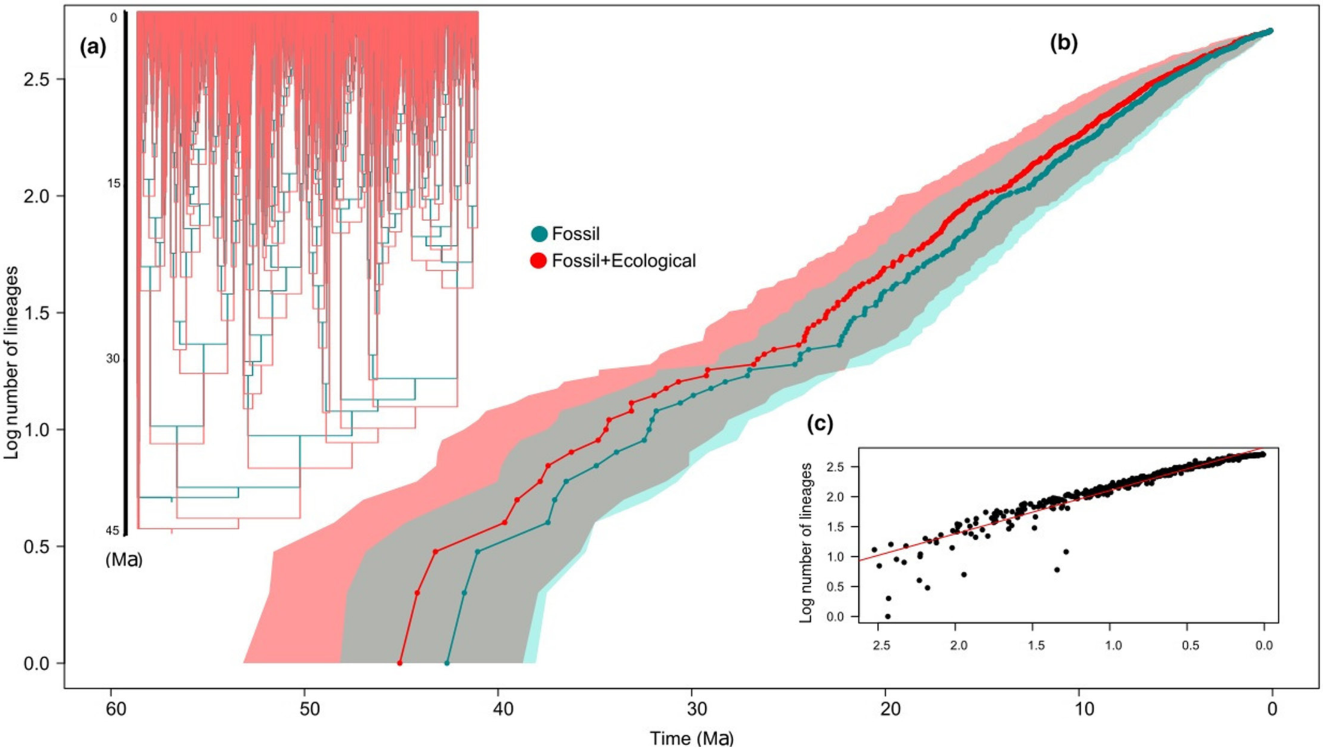

FIGURE 5 Comparison of divergence times estimated using Bayesian phylogenetic analysis in BEAST: (a) tree in turquoise estimated using three fossil calibrations (following Duellman et al., 2016) tree in red estimated with an additional Geological-Ecological Molecular Calibration (GEMC) using an estimate of the split within *Phytotriades auratus*; (b) number of lineages through time, shown in red and turquoise (with 95% credibility intervals) for the analyses calibrated as in (a), and (c) time difference in million years between the two sets of estimates in (b).

frog *Pristimantis urichi*, endemic to Trinidad and Tobago, is present in both islands, and allopatric populations lack genetic divergence (Jowers, Sánchez-Ramírez, et al., 2021). Similarly, the frog *Flectonotus fitzgeraldi* has low divergence across populations of Paria Peninsula, Trinidad, and Tobago (Smith et al., 2021). Thus, *P. auratus* is the only confirmed species in Trinidad to have diverged from counterpart populations through ascertained empirical geological data corresponding to an allopatric event.

Geological data indicate that after submerging of a section of the Paria-Northern range to circa 700 m above sea level and concomitant Paria Gulf sea opening, *Phytotriades* and *G. erectiflora* remained isolated on either side of the Gulf. At the estimated cut-off point of 700 m, cloud forest conditions diminished or did not exist, so neither the giant *G. erectiflora* nor *Phytotriades* could survive at lower altitudes over time. This mode of ecological vicariant speciation (Wiens, 2004) begins when environmental change creates conditions within a species' geographical range that are outside of its ancestral ecological niche. Yet, despite stepping-stone routes being put forward to explain rather challenging dispersal events across extreme barriers (Vences et al., 2003; Vidal et al., 2008; Vitt & Caldwell, 2014), the required ecological conditions for such a dispersal event would not have allowed the establishment of reproducing populations of both *G. erectiflora* and *P. auratus*. In addition, the vegetative reproduction of bromeliads through pups suggests that these giant plants were long established in the summits where they are found today, and that their propagation or presence in low-humidity conditions (i.e., ~700 m) would not be within the expected physiological tolerance of the species (Tonini et al., 2020). Remnant and ephemeral bromeliad populations might have persisted temporarily at slightly lower altitudes prior to going extinct over a relatively short timespan, as suggested by the geologically rapid rates of topographic lowering.

Indeed, continental separation leading to the split of lineages across oceans is not a cause of vicariance if these had already diverged by the time the landmasses separated. Sampling throughout a continuous range from Cerro Humo (most westernmost distribution: Venezuela) to Cerro del Aripo (easternmost distribution: Trinidad) would indeed help clarify the genetic isolation between populations on each side of the marine barrier to assess population divergence within regions prior to their split. However, in Venezuela this is not feasible, as only one population has been reported. In Trinidad, sampling of *P. auratus* at the two highest peaks (El Tucuche and Cerro del Aripo: Jowers et al., 2008) revealed no genetic divergence based on a *CYTB* fragment despite nearly 20 km of separation and presence of areas under 600 m in altitude between both populations, therefore suggesting recent gene flow. Due to the dispersal ability and the type of the barriers involved, which are critical in determining potential gene flow (Vitt & Caldwell, 2014), we examined such a scenario through ecological niche modelling. The latter indicates a continuous presence of bromeliads throughout the Paria Peninsula and Trinidad's Northern Range, suggesting no apparent reason for a discontinuous distribution for *Phytotriades* prior to the pull-apart of Paria basin.

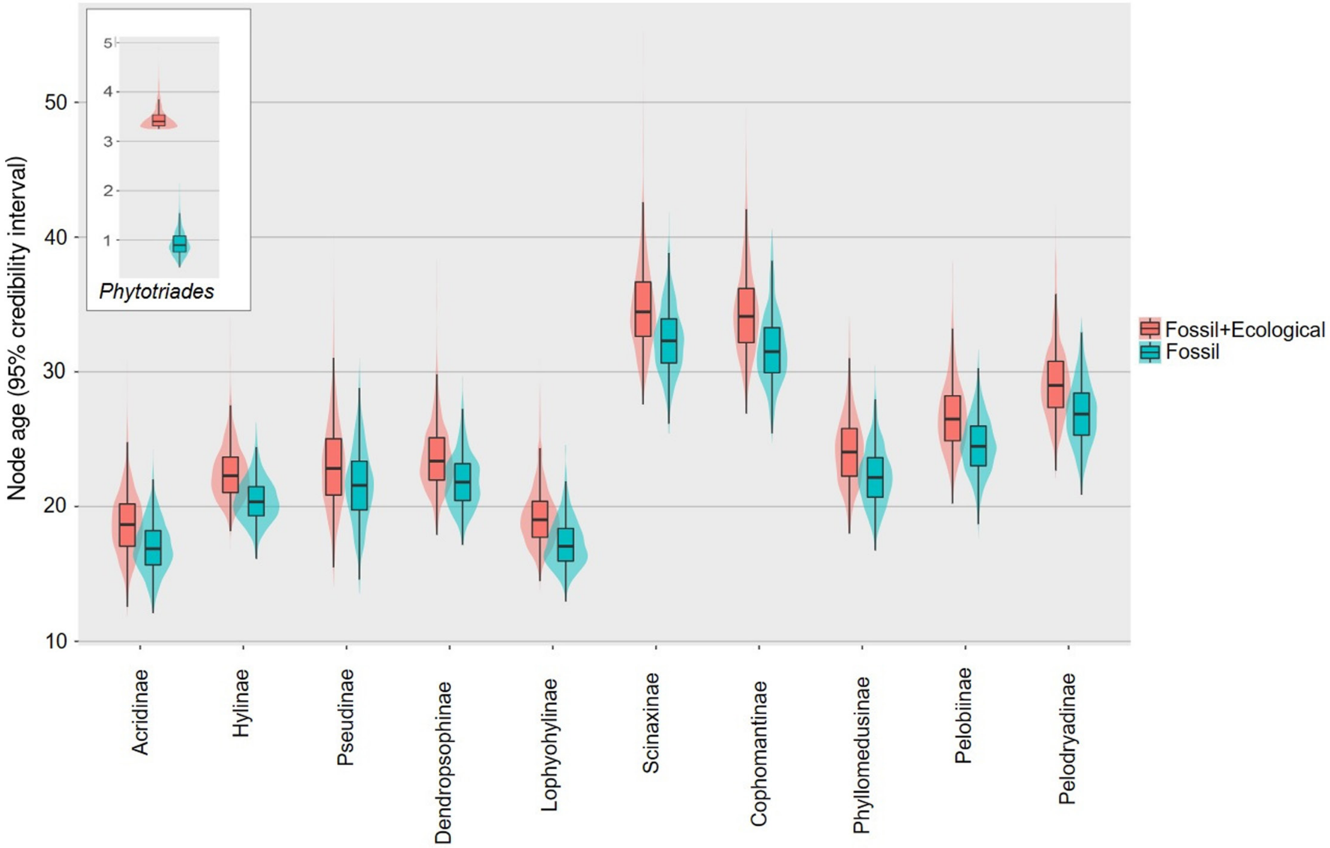

FIGURE 6 Estimated node of *Phytotriades* and all subfamilies ($n = 10$) in million years with 95% credibility intervals. Estimates in turquoise were obtained using three fossil calibrations following Duellman et al. (2016) estimates in red were obtained using the same three fossil calibrations plus a Geological-Ecological Molecular Calibration (GEMC) based on the split within *Phytotriades auratus*.

The geological and ecological niche models fit with having connectivity in the region, especially during the Pliocene, when the most suitable conditions occurred. A continuous presence of *G. erectiflora* was also supported in the older models. The available connective corridor in a relatively short distance suggests that these bromeliad and frog populations were previously connected. Given the evidence shown here, we can explain the presence of both populations through a vicariant event. Our data place the vicariance of the disjunct populations at 4.1 ± 0.85 Ma, a time when the altitude of the falling range would not sustain adequate conditions for the giant bromeliad and, therefore, would not allow for contact zones of either the plant host or the frog. Comparative analyses of the two trees confirm that estimates of recent divergence times among taxa could greatly benefit from biological data. A split at 0.94 Ma between the two populations of *Phytotriades* remains highly implausible for the ecological reasons given, and this dating is over three-fold younger than our most likely estimated divergence time.

The method of calibration proposed here has advantages over employing fossil data alone, as the latter may not be possible for recent evolutionary divergences (Ho et al., 2008). Taxa represented in the fossil record are usually unknown in terms of diagnostic characteristics at lower taxonomic levels; therefore, placing fossils within genera can rarely be done confidently. For example, the presumed ancestors of New World coral snakes (*Micrurus*) in Nebraska (USA) and France (Rage & Holman, 1984) date to the Miocene, but their validity as *Micrurus* is highly questionable (Zaher et al., 2019). Where possible, the biases and uncertainties of fossil data should be minimized in biogeographical analysis (Quental & Marshall, 2010).

Divergence times inferred using the combined fossil and GEMC calibrations are older than those inferred using the fossil calibrations alone, although the 95% credibility intervals overlap between our two sets of date estimates. Our analyses provide an age estimate for Arboranae of 41.7 Ma (95% CI 38.3–46.5 Ma) without the GEMC calibration and 44.4 Ma (95% CI 37.9–51.8 Ma) with it. These stand in contrast with previous estimates of 61.8 Ma (95% CI 57.5–66.1 Ma) (Duellman et al., 2016), ~67 Ma (Wiens, 2011), 71.1 Ma (Pyron & Wiens, 2013), and 70.7 Ma (Pyron, 2014) for the same node. Thus, including a single additional calibration has led to important differences in age estimates at the genus and subfamily levels, with changes of ~8%–9% in the timings of the clades. The age of Arboranae, while outside the scope of this work, will be resolved more confidently with further study. Compared with the use of deep fossil-based calibrations alone, including biological data for recent divergences can lead to improvements in the date estimates of shallower nodes in the tree.

5 | CONCLUSIONS

Our study has identified a rare and unique opportunity to assess the process of vicariance based on particular ecological, geological, and biological constraints. The incorporation of these vicariant timings to a molecular dating analysis of Arboranae resulted in subfamilies and genera being younger than previously thought. More generally, our study demonstrates that drawing on information from geological data has the potential to improve estimates of recent divergence times. We advocate for the use of ecological as well as geological data whenever possible in other study systems that can be used to date vicariant speciation with greater precision than without using such constraints. Examples such as plant–insect ecological associations might prove helpful in estimating the timing of recent evolutionary events.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The original alignment and phylogenetic trees are available from https://www.ugr.es/~ajesusmp/PhytoSuppMat/Trees.zip. Genbank accessions are available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

PERMITS

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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