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Comparative approaches to understanding speciation: a case study in *Xantusia* lizards

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ABSTRACT

The diversity of life forms that surround us formed through a variety of processes. Speciation researchers use a suite of comparative approaches to understand both the generalities and the variance underpinning this diversification. Here, we summarize these approaches and what they have taught us, and we then apply them to understand speciation in the night lizards *Xantusia*, a genus of small lizards found in the arid regions of western North America. Although *Xantusia* species span both a range of phenotypic and genetic divergence, we find that speciation in *Xantusia* appears to be a predictable consequence of divergence in extended periods of allopatry. We conclude by identifying possible areas of growth for comparative studies of speciation.

Keywords: ddRADseq; introgression; night lizards; phylogenetics; phylogeography; species delimitation

INTRODUCTION

When we scan across the tree of life, we can see the diversity of ways in which the tips—or the millions of species that constitute Earth's biodiversity—have formed. In some parts of the tree, species have diversified to eat different foods and/or to live in different habitats, yet their recent evolutionary origins make them genetically very similar (e.g. cichlids, pea aphids; Peccoud *et al.* 2009, McGee *et al.* 2020). In other clades, species seem ecologically interchangeable even though their closest ancestors trace back millions of years (e.g. *Batrachoseps* salamanders, rainforest skinks; Wake 2006, Singhal *et al.* 2018b). Additionally, a diversity of barriers maintain species, with some species seemingly separated only by assortative mating and others separated primarily by selection against hybrids (Christie *et al.* 2022). Additionally, even when barriers to gene flow separate species, some groups can experience extensive introgression even among distantly related species (Dagilis *et al.* 2022) whereas others remain completely genetically distinct. Zooming out to a broad-scale perspective, we find that species vary both in the rate at which they form (Cooney and Thomas 2021) and how they accumulate across geographical space, with some species achieving sympatry early in the divergence process (e.g. cichlids; McGee *et al.* 2020) and other species occurring across space as allopatric replacements (e.g. *Ensatina* salamanders; Wake 2006). Thus, just as we can see a diversity of forms across the tree of life, we can also see diversity in how they originated.

One way to understand the diversity of speciation processes is through the use of comparative analyses. By summarizing across many speciation events, comparative approaches can uncover trends and patterns across the myriad ways that speciation occurs. In this paper, we first briefly review the types of comparative analyses used in speciation research and what we have learned from them. Then, we explore a case study of comparative speciation in *Xantusia* lizards. Finally, we identify open challenges and directions for comparative speciation research.

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COMPARATIVE APPROACHES TO SPECIATION

Below, we describe five types of comparative studies that have been employed by speciation researchers, the types of questions these studies can help address, and some findings from these studies (Table 1).

Pairwise estimates of divergence

In this broad class of studies, metrics of differentiation—such as genetic, ecological, and morphological divergence—are assessed between taxon-pairs typically within the same taxonomic group (i.e. angiosperm plants, birds, fungi; Moyle *et al.* 2004, Giraud and Gourbière 2012, Freeman *et al.* 2023). Researchers typically also estimate multiple components of reproductive isolation (RI), including strength of mate choice and intrinsic selection against hybrids (Coyne and Orr 1989, Price and Bouvier 2002, Moyle and Nakazato 2010), as well as total RI from more indirect approaches (Singhal and Moritz 2013, Hamlin *et al.* 2020, Pulido-Santacruz *et al.* 2020). Correlations among differentiation and RI metrics can then help uncover the tempo and mode of divergence and determine the factors that initiate and 'complete' the speciation process.

As nicely reviewed by Matute and Cooper (2021), these studies have shown that both phenotypic divergence (Winger and Bates 2015, Freeman *et al.* 2023, Nosil *et al.* 2024) and RI (Coyne and Orr 1989, Sasa *et al.* 1998, Moyle *et al.* 2004) often scale with increasing genetic divergence. Further, in many clades, postzygotic isolation evolves more predictably than prezygotic isolation (Christie and Strauss 2018, Sianta and Kay 2021). However, these relationships between RI and time are not necessarily monotonic—RI can also quickly accrue over short spans of divergence (Roux *et al.* 2016, Singhal and Bi 2017, Peñalba *et al.* 2019)—so-called 'tipping points' of speciation (Nosil *et al.* 2017). Other studies have evaluated the relative importance of ecology in driving speciation, with several studies suggesting that ecological divergence can predict genetic divergence and/or RI (Funk *et al.* 2006, Shafer and Wolf 2013) while others argue that much of allopatric speciation occurs in the absence of divergent selection (Anderson and Weir 2022).

Comparative geography of speciation

The arrangement of species in space both reflects the probable geographical mode of speciation (i.e. allopatric, parapatric, sympatric) and the controls influencing coexistence between closely related species. Another class of comparative studies considers patterns of species overlap within and across clades to understand both the geography of speciation and the factors determining species coexistence (Losos and Glor 2003, Weber and Strauss 2016).

These studies have shown that even closely related species—like sister taxa—often have high degrees of range overlap; anywhere from 30% to 90% of sister species are found in sympatry across diverse taxa such as plants (Anacker and Strauss 2014, Grossenbacher *et al.* 2014, Christie and Strauss 2018), butterflies (Rosser *et al.* 2015), and fish (Glass *et al.* 2023). A high degree of overlap might suggest that many species either originate in sympatry (Fitzpatrick *et al.* 2009) or achieve sympatry relatively quickly after divergence (Anacker and Strauss 2014). However, in other groups, secondary sympatry can take millions of years to achieve (Weir and Price 2011, McEntee *et al.* 2018, Pigot *et al.* 2018), and is most likely when taxa are high-dispersing, phenotypically divergent, and reproductively isolated (Weir and Price 2011, Pigot and Tobias 2013, 2015, Anacker and Strauss 2014, Cooney *et al.* 2017b, McEntee *et al.* 2018, Pigot *et al.* 2018). Finally, species range overlap—via either reinforcement (Coyne and Orr 1989) or character displacement (Anderson and Weir 2021)—can drive increased reproductive or ecological divergence, further isolating the species.

Comparative phylogeography and biogeography

Comparative phylogeography and biogeography studies focus on species in a common geographical arena and look for concordance in both space and time in phylogeographical breaks or species borders. They use genetic data to infer the demographic changes that accompany speciation, such as characterizing how population subdivision can precipitate lineage divergence and how diverging lineages interact via gene flow. These studies often also integrate modelling of species ranges through time to better understand how changing distributions might affect lineage

Table 1. Comparative methods that can be used to understand speciation.

Method	Description	Example question
Pairwise estimates of divergence	Comparing extent of reproductive isolation and/or genetic, morphological, and ecological divergence among lineages	What is the tempo at which reproductive isolation evolves?
Comparative geography	Comparing patterns of range overlap across closely related lineages	How does the extent of ecological differentiation affect transition rate to secondary sympatry?
Comparative phylogeography	Understanding the role of history and demography in structuring patterns of genetic diversity across lineages found in a common geographical arena	How does historical stability affect lineage persistence?
Macroevolutionary analyses	Understanding broad-scale patterns of diversification across traits, clades, and biogeographical regions	Which organismal traits predict variation in speciation rate?
Comparative genomics	Investigating patterns of genetic divergence across closely related lineages	Are patterns of genomic differentiation predictable across species?

divergence (Graham *et al.* 2006, Carnaval *et al.* 2009, Smith *et al.* 2014, Yamasaki *et al.* 2020). In so doing, these approaches help understand the geological and historical factors that help promote and maintain lineage formation (Bermingham and Moritz 1998, Edwards *et al.* 2022).

Here, we focus on a few themes that have emerged from the rich and long history of this field (Avise 2000). First, rarely do phylogeographical lineages separated by biogeographical or habitat barriers follow the same evolutionary trajectory. Across a single barrier, codistributed lineages typically span a range of divergence times and show variance in the location of parapatric boundaries (Avise 1992, Whinnett *et al.* 2005, Soltis *et al.* 2006, Rosenblum and Harmon 2011, Leaché *et al.* 2020). This variance could be because species experience these barriers differentially depending on their biology (Papadopoulou *et al.* 2009, Fouquet *et al.* 2012) and/or the cyclical nature of many of these barriers (Dynesius and Jansson 2000). Cyclical barriers can result in gene flow eroding divergence among isolated populations at different time points and to different strengths across their history (Smith *et al.* 2014). Second, areas that promote greater population persistence—such as geographical regions that have been more stable through time—can promote the persistence and build-up of species (Graham *et al.* 2006, Carnaval *et al.* 2009, Smith *et al.* 2014, Yamasaki *et al.* 2020). Conversely, unstable regions—such as regions of high environmental harshness—often show evidence of greater initiation of speciation but lower rates of completion (Botero *et al.* 2014, Dantas-Queiroz *et al.* 2023). Third, gene flow is fairly common among diverging lineages (Winger 2017, McLaughlin *et al.* 2020), even in cases where lineages are not currently overlapping (Peñalba *et al.* 2019, Pulido-Santacruz *et al.* 2020). This finding highlights that species geographical arrangements shift through time, allowing gene flow even between currently allopatric lineages. Finally, despite a rich verbal history in phylogeography for the potential role of bottlenecks and founder events in speciation (Barton and Charlesworth 1984), demographic modelling of population sizes through time suggests that genetic drift is unlikely to be a major force in triggering lineage divergence (Knowles 2001).

Macroevolutionary analyses of species richness and diversification

Macroevolutionary studies are inherently comparative and thus address a wide range of questions about the speciation process. Here, we focus on two types of macroevolutionary studies. The first reconstructs trait evolution and biogeographical area across clades to understand how transitions in traits and landscapes correlate with shifts in diversification patterns (Hughes and Eastwood 2006, Whittall and Hodges 2007, Matzke 2014). The second endeavours to explain diversity gradients in species richness and speciation rate found across traits, geographical distributions, and the tree of life (Rabosky 2016, Schluter and Pennell 2017). Note that some macroevolutionary studies measure diversification (or the net balance between speciation and extinction) whereas others focus on speciation only.

A few themes emerge from these studies. First, higher diversification rates often fail to predict greater species richness (Tietje *et al.* 2022), due to factors including clade age (McPeek and Brown 2007), ecological limits (Ricklefs 2007, Rabosky 2009), and phylogenetic or geographical scale (Hutter *et al.* 2017). This is perhaps clearest in studies of the Latitudinal Diversity Gradient, or the pattern of increasing species richness as one moves from the polar caps to the equator (Mittelbach *et al.* 2007). In many taxa, the gradient in species richness across latitude is not mirrored by a gradient in speciation rate (Jetz *et al.* 2012, Economo *et al.* 2018, Rabosky *et al.* 2018, Igea and Tanentzap 2020, Title *et al.* 2024), but see Cardillo (1999). In fact, this pattern is often inverted, with higher rates of speciation in regions with lower species diversity (Harvey *et al.* 2020). Second, several traits correlate with variation in species richness and/or diversification rate, such as asexuality in reptiles, mutation rate in birds, seed size in plants, and male-biased sexual selection in birds (Lanfear *et al.* 2010, Igea *et al.* 2017, Cally *et al.* 2021, Moreira *et al.* 2021). However, these patterns are often inconsistent even within the same clade (Helmstetter *et al.* 2023). In fact, in many groups, organismal and environmental traits rarely explain a significant portion of the variation in speciation rate (Title *et al.* 2024). This holds true even when traits with direct relevance to the speciation process are considered, such as the rate at which song divergence in birds evolves (Freeman *et al.* 2022), the rate at which postzygotic isolation evolves in birds and flies (Rabosky and Matute 2013), or the rate at which isolated populations form in birds and lizards (Singhal *et al.* 2018a, 2022, Wacker and Winger 2024) but see Harvey *et al.* (2017). Finally, perhaps some of the strongest predictors of speciation rate are not trait identity but the rates at which these traits evolve (Rabosky *et al.* 2013, Igea *et al.* 2017, Beltrán *et al.* 2021, Cooney and Thomas 2021). While theory predicts this correlation—phenotypic change can precipitate both ecological divergence and RI, after all—these correlations might be technical artefacts resulting from comparing two time-varying processes (Harmon *et al.* 2021).

Comparative genomics of speciation

With the increasing ease of sequencing whole genomes for diverging species, a new class of studies have emerged: the comparative genomics of speciation. By sequencing whole genomes for multiple individuals across multiple species, these studies primarily address two major classes of questions. First, what are the relative roles of genomic processes such as selection, introgression, and genome structure in driving divergence across species? These studies have shown that loci putatively linked to adaptive phenotypes often exhibit the greatest genetic differentiation across species (Riesch *et al.* 2017, Edelman *et al.* 2019, Todesco *et al.* 2020) and that adaptation can make divergence with gene flow more likely (Egan *et al.* 2008, Kautt *et al.* 2020). Further, as has long been appreciated by plant biologists (Grant 1971), these studies illustrate that introgression is common, even among species that are deeply diverged (Singhal *et al.* 2021, Barley *et al.* 2022). Further, this introgression can help fuel speciation and species divergence (Lamichhaney *et al.* 2018, Meier *et al.* 2023, Rosser *et al.* 2024), especially when the introgressed loci are under strong selection. Finally, these studies have identified an important role for structural variation in creating and maintaining species boundaries (McGee *et al.* 2020, Todesco *et al.* 2020).

Second, can we predict the heterogeneity in both genomic divergence and introgression across interacting species pairs? As lineages form, divergence across the genome is typically uneven, and if lineages are exchanging genes, levels of gene flow across the genome are also typically uneven. To address this question, researchers measure the repeatability of genomic divergence and introgression across multiple species pairs. Thus far, studies have found modest (Ravinet *et al.* 2016, Delmore *et al.* 2018, Meier *et al.* 2018) to strong evidence for predictability (Van Doren *et al.* 2017, Vijay *et al.* 2017, Langdon *et al.* 2022). Predictability can arise due to direct selection (Renaut *et al.* 2013, Pereira *et al.* 2016, Nouhaud *et al.* 2022), background selection (Burri *et al.* 2015, Stankowski *et al.* 2019, Langdon *et al.* 2022), selective introgression (Edelman *et al.* 2019, Rossi *et al.* 2024), and/or sorting of ancestral variation (Irwin *et al.* 2016) but see Schluter and Rieseberg (2022). Finally, these studies show that repeatability increases as species divergence becomes deeper (Singhal and Bi 2017, Delmore *et al.* 2018, Stankowski *et al.* 2019), as verbally predicted by Burri *et al.* (2015).

CASE STUDY: SPECIATION IN *XANTUSIA* LIZARDS

Introduction to system

Here, we explore the potential of comparative approaches to speciation through a study of the night lizards *Xantusia* (Xantusiidae). These small, secretive lizards are split into 14 recognized species, which are distributed across arid regions of the southwestern USA and México (Uetz *et al.* 2023). *Xantusia* species fall into one of two habitat specialist types (Fig. 1). The rock ecomorph is typically found among rock-crevice and boulder habitats, and these species have longer limbs and digits, flatter and longer bodies, and more colourful patterning than the plant ecomorph, which is often found within decaying plant material or under bark across yucca, agave, and other plants (Bezy 2019). *Xantusia* has transitioned between these two morphs at least seven times (Noonan *et al.* 2013), and convergence across these transitions is high. In fact, morphological data suggested two rock forms (*X. bolsonae* and *X. henshawi*) were closely related until genetic data revealed they are distant relatives (Webb 1970). Further, some relatively recent species splits are associated with a transition in ecomorph type (e.g. *X. sierrae*), posing a natural contrast between speciation events resulting in two species of the same ecomorph ('nonecological' speciation) and those that result in species of different ecomorphs ('ecological' speciation) (Schluter 2009).

In this work, we explore patterns of *Xantusia* speciation using a population genetic dataset of 140 individuals collected across 11 nominal species. Before we can understand speciation in *Xantusia*, we must first understand what the species units are. *Xantusia* lizards are notable for their extremely low dispersal rates—in fact, some individuals are repeatedly recaptured over years from the same locality (Zweifel and Lowe 1966) and natal dispersal is estimated at just 4 m in *X. vigilis* (Davis 2011, Davis *et al.* 2011). As might be expected given their low vagility, many species are composed of deeply structured, putatively morphologically cryptic phylogeographical lineages (Lovich 2001, Sinclair *et al.* 2004). *Xantusia* also tend to occur in patchy distributions across the landscape (Zweifel and Lowe 1966), as their presence is thought to be dictated (in part) by suitable habitat. Species thus consist of populations in high density, separated by large swathes of inhospitable habitat (Bezy 2019). Low dispersal and geographical patchiness make it difficult to identify the boundary between populations and species. To address these challenges, we use genetic analyses—i.e. phylogenetic inference, population clustering, and isolation-by-distance tests—to define species boundaries in *Xantusia*.

Having delimited species in this group, we then apply approaches from comparative phylogeography, comparative geography, macroevolutionary studies, and pairwise divergence studies to understand the tempo, demography, and geography of *Xantusia* speciation. (We wait for a *Xantusia* reference genome before we apply the approaches from comparative genomics described above.) We might expect patterns to vary across type of speciation—i.e. 'ecological' versus 'nonecological' speciation—and based on the age of a species pair (Stankowski and Ravinet 2021, Bolnick *et al.* 2023). For example, ecological speciation is typically thought to be more robust to gene flow than nonecological speciation (Anderson and Harmon 2014), given that selection to different environments can counteract the homogenizing effects of gene flow. Further, gene flow is typically thought to decline to zero as species accumulate divergence and barriers to reproduction (Sobel and Chen 2014). Thus, we might predict that gene flow would be more common among pairs that

Figure 1. *Xantusia* species consist of two ecomorphs: plant-specialist and rock-specialist. The rock ecomorph has longer limbs, a flatter body, and more colourful patterning than the plant ecomorph, and there have been multiple transitions between the two ecomorphs (see Supporting Information Fig. S5). Images by Julie Johnson of Life Science Studios.

share the same ecomorph and that are younger. We test these expectations to better understand the origins of this clade.

MATERIALS AND METHODS

Sampling, genetic data collection, and processing

We sampled 140 individuals from 11 of the 14 recognized species in *Xantusia* (Uetz *et al.* 2023). Where possible, we sampled broadly across species ranges to capture any phylogeographical structure within species (Lovich 2001, Sinclair *et al.* 2004, Leavitt *et al.* 2007); number of samples collected per species ranged from one to 76 (median: seven individuals). In addition, we sampled three individuals in the genus *Lepidophyma* to use as an outgroup. In total, we sampled 143 individuals (Supporting Information Table S1), most of which derived from natural history museum collections and nine of which were newly collected for this study.

From each individual, we extracted DNA and then prepared double-digest restriction-site associated DNA (ddRAD) libraries following Peterson *et al.* (2012). Briefly, we digested DNA using MspI and SbfI, size-selected from 415 to 515 bp, and then prepared uniquely barcoded ddRAD libraries with an 8-bp unique molecular identifier (UMI) ligated to the cut site. Equimolar amounts of libraries were then pooled and sequenced on a single 100SE lane of an Illumina NovaSeq 6000 at the Vincent J. Coates Genomics Sequencing Lab, QB3 Genomics, UC Berkeley.

After trimming the 8-bp UMI, we demultiplexed reads using stacks v.2.64 (Rochette *et al.* 2019). We then used ipyrad v.0.9.93 to assemble the data and call variant sites (Eaton and Overcast 2020). We clustered the data at 85% similarity, requiring a minimum depth of six for variant-calling and two or fewer alleles per site. We then generated a second assembly that excluded the outgroup, keeping all other parameters the same.

Additionally, we collated previously published mitochondrial DNA (mtDNA) data for the *ND4* gene (Lovich 2001, Sinclair *et al.* 2004, Leavitt *et al.* 2007). We included individuals from *Cricosaura typica* and the genus *Lepidophyma* as outgroups. After downloading these data from GenBank, we aligned them using mafft v.7.487 (Katoh *et al.* 2009). Because these are entirely coding sequences, we manually checked the alignment for the absence of indels using Geneious v.2022.2.2 (Kearse *et al.* 2012).

Lineage delimitation

Our comparative speciation analyses required us to identify the putative lineages in *Xantusia*. We define lineages as independently evolving, genetically cohesive units. To do so, we conducted both phylogenetic and population structure analyses to understand how genetic diversity in this genus is apportioned into lineages. First, using the outgroup assembly, we concatenated all ddRAD loci assembled in > 50% individuals. We then inferred an individual phylogeny in IQ-TREE v.2.2.0 (Minh *et al.* 2020), using ModelFinder to automatically determine and apply the best-fitting model of sequence evolution (Kalyaanamoorthy *et al.* 2017). We additionally calculated nodal support through Shimodaira–Hasegawa approximate likelihood ratio tests (SH-aLRT) (Anisimova and Gascuel 2006). Then, we used IQ-TREE to infer the mtDNA topology; here, we partitioned the alignment by coding position and used PartitionFinder to automatically identify the best partition and molecular evolution scheme.

We next inferred population structure across the genus using two approaches. We first created 10 filtered variant sets that randomly sampled one variable site per locus, requiring sites to be > 50% complete and with a minor allele count ≥ 2 (Linck and Battey 2019). Using one of these filtered variant sets, we mapped individuals in genotypic space using a principal component analysis (PCA), as implemented in glPCA in adegenet v.2.1.10 (Jombart 2008). Here, missing values are replaced with mean allele frequency values. Our phylogenetic analyses and PCA results (see Results) revealed that *Xantusia* consists of three major clades or clusters, respectively. For each of these three groups, we ran ADMIXTURE v.1.3.0 (Alexander *et al.* 2009) across all 10 filtered variant sets, allowing the number of genetic clusters (K) to vary from 1 to 12. We estimated the cross-validation (CV) score to determine the best-fitting K value for each set of runs.

Our phylogenetic and population structure analyses revealed multiple phylogeographical lineages within currently recognized species, all of which had been identified in previous studies (Lovich 2001, Sinclair *et al.* 2004, Leavitt *et al.* 2007). Here, we treated these lineages as putative species-level taxa alongside currently recognized species-level taxa. To explore the evolutionary distinctiveness of these species-level taxa, we investigated how isolation-by-distance patterns accrue between taxa. If taxa are acting as independent evolutionary units, they would experience no or reduced gene flow between them, resulting in a break in genetic continuity across geographical space (Good and Wake 1992, Hausdorf and Hennig 2020). We calculated genetic divergence across space as pairwise F_{ST} between individuals (Reich *et al.* 2009) and assessed the significance of isolation-by-distance using Mantel tests as implemented in the R package vegan v.2.6 (Dixon 2003). Given our study did not aim to formally delimit and revise *Xantusia* taxonomy, we refrained from applying more statistical approaches such as multispecies coalescent delimitation methods. Ultimately, we found equivocal evidence for the genetic distinctiveness of two recognized species and two phylogeographical lineages. Because the recognized species had been previously diagnosed as morphologically distinct and all species-level taxa showed some evidence for being genetically distinct, our subsequent analyses implemented an operational taxonomy that included all identified recognized species and phylogeographical lineages.

Finally, for this operational taxonomy, we inferred a taxon-level phylogeny using SNAPP v.1.6.1 (Bryant *et al.* 2012), a multispecies coalescent approach that uses single nucleotide polymorphism (SNPs) to infer a species tree. Because we lacked the appropriate outgroup sampling to use fossil calibrations (Noonan *et al.* 2013), we instead inferred relative divergence times. We set the mutation rates u and v to 1.0, and for the species tree prior we used a gamma distribution ~ (1, 250). We ran four independent analyses for 1 million generations each, sampling every 100 generations. We assessed for convergence by visualizing the log-likelihood Markov chain Monte Carlo (MCMC) trace and checking effective sample size values. Then, we combined the posterior probabilities for each run using LogCombiner and used the combined posteriors to assemble a maximum clade credibility (MCC) tree in TreeAnnotator, after discarding the first 20% of samples as burn-in.

Mapping ecomorphological transitions

A key goal of our study is to compare speciation patterns across ecomorphs, so we first characterized the evolution of 'plant' and 'rock' ecomorphs in *Xantusia*. We coded taxa by their ecomorph type based on sources from the primary literature (Sinclair *et al.* 2004, Leavitt *et al.* 2007, Noonan *et al.* 2013). We then mapped the evolution of the ecomorphs across the species tree using the stochastic character mapping approach implemented in phytools v.2.1-1 (Huelsenbeck *et al.* 2003, Revell 2024). Here, we used the equal-rates model, in which the transition rates between the two morphs were equal; other models were less good fits to the data (Supporting Information Table S2). We ran simulations for a posterior sample of 1000 species trees and ran 1000 simulations across the consensus species tree.

Comparative speciation of *Xantusia*

We conducted a series of analyses to characterize the tempo, demography, and geography of speciation in *Xantusia*. In all cases, we compared patterns between all possible species pairs within each of the three major clades. First, we calculated estimates of genomic divergence (F_{ST}, d_{xy}) on the full variant set and additionally calculated d_{xy} on the mitochondrial data (Nei and Li 1979, Reich *et al.* 2009).

Then, to understand the demography of speciation in *Xantusia*, we conducted four analyses, each of which offers differing resolution onto the temporal and spatial extent of gene flow. First, we fit demographic models following the approximate Bayesian computation (ABC) approach implemented in DILS (Fraïsse *et al.* 2021). DILS generates simulated data under current isolation (strict isolation, ancient migration) versus ongoing migration (isolation-with-migration, secondary contact) divergence models under both constant and changing population sizes. It then uses a Random Forest classifier to determine which model best fits the empirical data. Ultimately, this analysis estimates the 'probability of migration', or the likelihood that there is ongoing gene flow between the two lineages. Second, we mapped the population clustering results across geographical space. Individuals can appear to be genetically admixed even when they are not hybrids (Lawson *et al.* 2018), but putatively admixed individuals are more probably the result of hybridization if they occur near the geographical borders between their parental lineages. Third, we compared patterns of mitochondrial to nuclear lineage identity to determine if there is any evidence for mitochondrial capture (Toews and Brelsford 2012). Mismatches in clade identity between mitochondrial and nuclear markers can simply result from incomplete lineage sorting. However, similar to our population clustering analysis, introgression is a more likely scenario if these mismatched individuals occur at the geographical boundaries between their mitochondrial and nuclear donor lineages. Finally, we used Dsuite v.0.5 to infer the D-statistic across all possible trios (Malinsky *et al.* 2021). Introgression involving ancestral branches can lead to multiple descendant lineages showing evidence for introgression; we accounted for this possibility by summarizing our results with the f-branch statistic (Malinsky *et al.* 2018).

Additionally, to understand the spatial dynamics of speciation in *Xantusia*, we analysed geographical range overlap in the genus. For each operational taxonomic unit (OTU), we approximated geographical range as the alpha hull spanning all points (alphahull v.2.5; Pateiro López and Rodríguez Casal 2010). Then, we measured pairwise range overlap and geographical distance between geographical ranges.

Data analysis and visualization

All scripts used in data analysis and visualization are available at https://github.com/singhal/xantusia. Our scripts used Python v.3.9.12 and R v.4.3.1, including the R packages ape v.5.7, cowplot v.1.1.3, dplyr v.1.1.4, ggplot2 v.3.4.4, and sf v.1.0 (Paradis *et al.* 2004, Pebesma 2018, Wickham *et al.* 2019, Wilke *et al.* 2019).

RESULTS AND DISCUSSION

Lineage delimitation

We successfully collected an average of 513 kb across 6338 loci across our 140 in-group individuals. These genetic data showed that *Xantusia* consists of three major clades (Fig. 2; Supporting Information Fig. S1): one consists of species distributed in México (*X. gilberti*, *X. sherbrookei*, *X. bolsonae*, *X. sanchezi*, *X. extorris*), another consists of *X. henshawi*, and the final consists of species distributed mainly in the USA (*X. bezyi*, *X. wigginsi*, *X. arizonae*, *X. sierrae*, *X. vigilis*). The relationship among these three clades is uncertain; our nuclear and mitochondrial analyses differ with respect to the placement of the *X. henshawi* clade being either sister to the *vigilis* clade (nuclear data) or sister to the México clade (mtDNA) (Fig. 2; Fig. S2).

Both our nuclear and mitochondrial phylogenetic trees and population clustering analyses revealed the presence of deep structure within several recognized *Xantusia* species, confirming previous results based on fewer markers (Fig. 2; Supporting Information Fig. S2; Lovich 2001, Sinclair *et al.* 2004, Leavitt *et al.* 2007). *Xantusia henshawi*—a species that ranges from southern California to northern Baja California—consists of three deep lineages; geographical borders between these lineages fall partially along fault lines (Fig. S8; Lovich 2001). Isolation-by-distance (IBD) patterns suggest that these monophyletic lineages are acting as independent evolutionary units (Fig. S3).

The wide-ranging species *X. vigilis* is paraphyletic and consists of five lineages structured by geography (Fig. 2): the San Jacinto (SJ), Yucca Valley (YV), Owens Valley (OV), Kern Canyon & Antelope Valley (KCAV), and Eastern Mojave (EM) lineages. Names here follow Leavitt *et al.* (2007). Although the best-fitting cluster analysis collapsed the *X. vigilis* SJ and YV lineages into a single cluster, they are reciprocally monophyletic (Fig. 2), and IBD patterns are discontinuous across the two lineages (Supporting Information Fig. S3). Similarly, while *X. vigilis* OV and *X. sierrae* belong to a single genetic cluster (Fig. 2), IBD patterns suggest they are evolutionarily independent (Fig. S3). The relationship between *X. vigilis* KCAV and EM is more ambiguous. They are not reciprocally monophyletic and share a common IBD pattern across space (Fig. S3) but fall into distinct genetic clusters (Fig. 2). Finally, although the genetic clustering results grouped together the species *X. bezyi* and *X. wigginsi*, these are not sister species, and IBD patterns suggest they are evolutionarily distinct (Fig. S3).

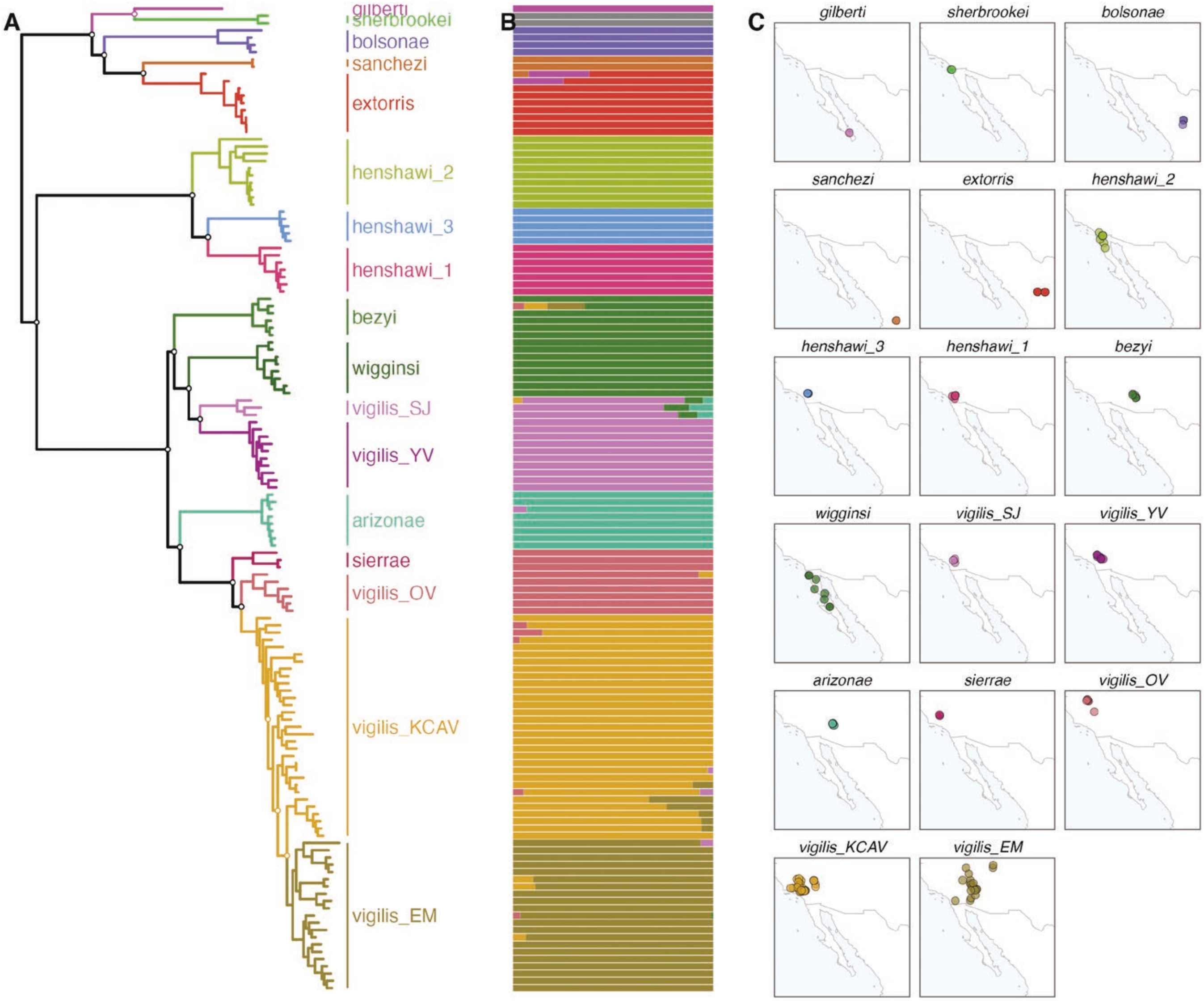

Figure 2. Phylogenetic and population structure of the operational taxonomic units (OTUs) in the genus *Xantusia*. A, the nuclear phylogeny was inferred with maximum likelihood on an alignment of 6233 loci (504 kb); tree rooted with *Lepidophyma* (not shown). Species-level and interspecific nodes with support values > 95% are marked with white circles; support values measured using approximate SH likelihood ratio tests. B, genetic clustering was inferred using ADMIXTURE after splitting the genus into three clade-level groups (see Supporting Information Fig. S1); results are combined here for visualization. C, maps illustrating the sampling distribution for each OTU.

Populations within low-vagility species are often genetically quite divergent. When species also occupy patchy distributions, geographical gaps between such populations can be hard to distinguish from species range boundaries. Thus, in low-dispersing, patchily distributed species like *Xantusia*, determining the genetic transition between population and species can seem arbitrary (Huang 2020). We see evidence for these challenges in the equivocal support for the evolutionary distinctiveness of *X. vigilis* KCAV and EM. However, for the majority of *Xantusia* taxa, the genetic analyses—i.e. phylogenetic reconstructions, genetic structure inference, and IBD patterns—provided a coherent perspective on species boundaries for the majority of *Xantusia* taxa. Indeed, within OTUs, we find strong evidence for continuous IBD (Supporting Information Fig. S4), suggesting that *Xantusia* lizards are moving enough among habitat patches to maintain genetic cohesion (Prates *et al.* 2022). However, very few OTUs share parapatric range boundaries or overlap (see Fig. 6B). So, while our results indicate that our operational taxonomy is robust, we do not yet know if these OTUs have evolved sufficient reproductive barriers and/or ecological divergence to stably coexist with congenerics.

Mapping ecomorphological transitions

The species tree for *Xantusia* recapitulated most of the major patterns seen in the individual-based nuclear and mitochondrial tree (Fig. 3), with most discrepancies mapping to nodes with low branch support. Onto this tree, we used stochastic mapping to infer ecomorph transitions, identifying an average of 6.7 changes in ecomorph across the *Xantusia* phylogeny (Supporting Information Fig. S5). The most likely ancestral state was the plant ecomorph (Sinclair *et al.* 2004, Noonan *et al.* 2013), and in the most common mapping, there were an

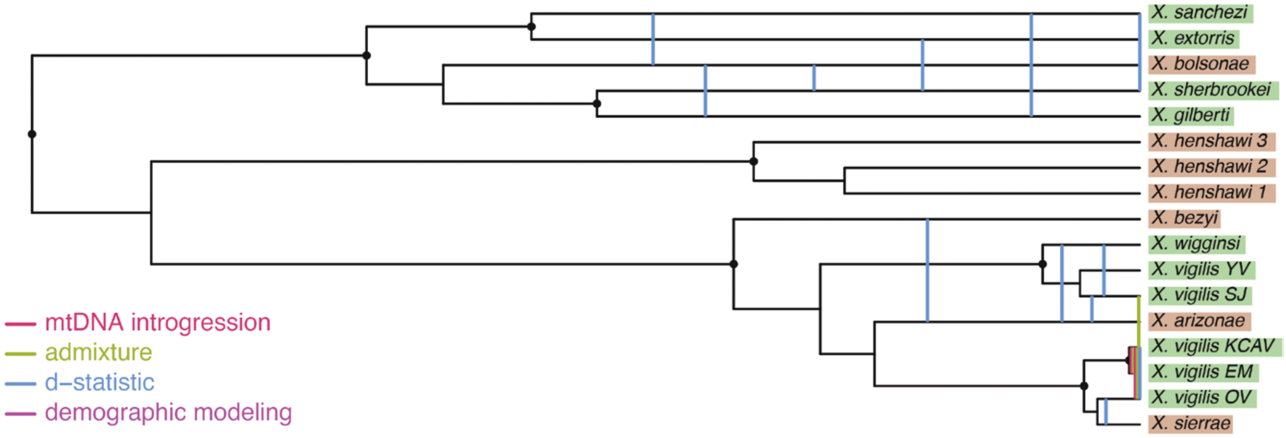

Figure 3. Evidence for hybridization and introgression across *Xantusia* based on four analytical approaches: identifying putative cases of mtDNA introgression, pinpointing probably admixed individuals based on genetic population clustering, using the D-statistic approach, and applying demographic modelling. Lines connect operational taxonomic units (OTUs) between which hybridization or introgression was detected; line colour indicates under which approach. Phylogeny is the species tree inferred with SNAPP; nodes labelled with points have posterior probability ≥ 0.95. Tips are coloured by the ecomorph identity of OTUs: plant-specialist (green) versus rock-specialist (brown). For the D-statistic results, we do not map introgression events involving ancestral branches. Note that absence of evidence for introgression should not be treated as a true absence; with greater geographical sampling or other analytical approaches, we might uncover other introgression events. Introgression as inferred by the D-statistic is particularly common, even among nonsister species, and some species comparisons show evidence for introgression across multiple approaches (e.g. *X. vigilis* KCAV and EM).

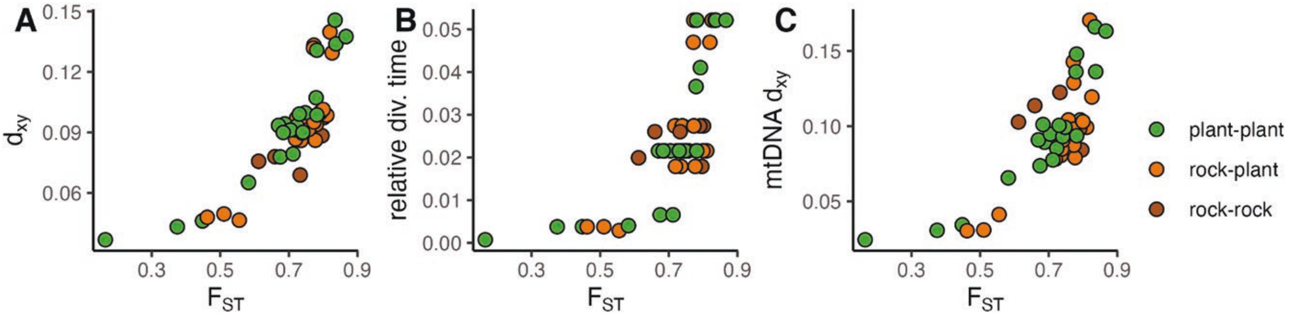

Figure 4. Patterns of genetic isolation and divergence across *Xantusia*. Genetic divergence—as measured by (A) F_{ST} and d_{xy}, (B) F_{ST} and relative divergence time, and (C) F_{ST} and mtDNA d_{xy}—is highly correlated across pairwise operational taxonomic unit (OTU) comparisons. We only show comparisons between OTUs in the same major clade (see Supporting Information Fig. S1); all comparisons are coloured by the habitat types of the OTU pair.

estimated five transitions from the plant to rock ecomorph and zero transitions from the rock to plant ecomorph. While most transitions are inferred to be relatively deep in the clade, one transition maps to the relatively recent split between *X. vigilis* OV and *X. sierrae*. Bezy (2019) suggested that *Xantusia* populations moved from living in highly flammable plants to rock crevices during extended periods of fire regime. This shift in habitat then precipitated the convergent evolution of the rock form. Because ecomorph transitions occurred across a range of phylogenetic depths in *Xantusia* (Fig. S5), these habitat shifts probably occurred across multiple distinct time points in the history of the genus.

Comparative speciation of *Xantusia*

Speciation dynamics vary across *Xantusia*. Some speciation events are associated with a shift in ecomorph type (i.e. *X. vigilis* OV and *X. sierrae*) while others are between recognized species of the same ecomorph (i.e. *X. sanchezi* and *X. extorris*), and others are between putatively cryptic OTUs (i.e. *X. henshawi* 1 and *X. henshawi* 2). These splits span a wide range of genetic divergence, with F_{ST} values ranging nearly continuously four-fold from 0.16 to 0.86 (Fig. 4), with no evidence that genetic divergence between ecomorphs or between putatively cryptic OTUs is distinct from that between other species pairs.

Although we are increasingly finding evidence for gene flow during divergence (Marques *et al.* 2019), *Xantusia* is different. Demographic modelling provided no evidence that any of these species are experiencing ongoing migration (Fig. 5); the highest probability of migration was only $P = 0.20$. In similar studies in other systems, taxa transition from having a high probability of ongoing migration to low over a narrow range of genetic divergence—e.g. from a net genetic divergence of 0.5% to 2% (Roux *et al.* 2016, Peñalba *et al.* 2019, Fraïsse *et al.* 2021). In *Xantusia*, however, the probability of migration did not vary as a function of either ecological or genetic divergence between taxa. These results suggest that *Xantusia* species were mostly isolated

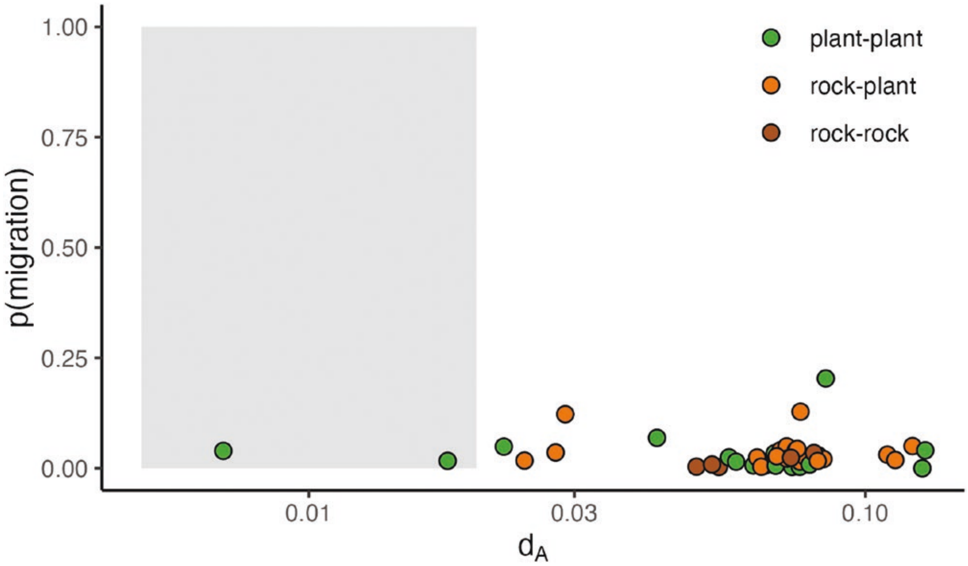

Figure 5. Likelihood of ongoing migration between pairwise operational taxonomic unit (OTU) comparisons. We only show comparisons between OTUs in the same major clade (Supporting Information Fig. S1); all comparisons are coloured by the habitat types of the OTU pair. The grey box spans the 'grey zone' of speciation originally identified by Roux *et al.* (2016), or the range of genetic divergence in which populations transition from experiencing ongoing migration to complete isolation. We find no such transition; ongoing migration was unlikely across all OTU pairwise comparisons.

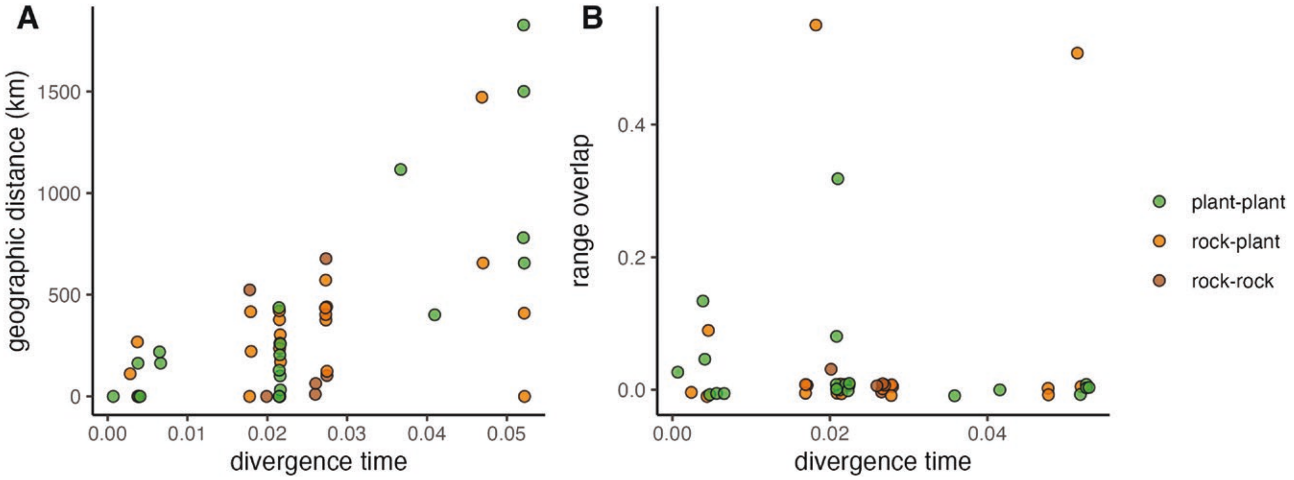

Figure 6. A, minimum geographical distance; and B, range overlap by relative divergence time between pairwise comparisons of *Xantusia* operational taxonomic units (OTUs). Points are slightly jittered for visualization. We only show comparisons between OTUs in the same major clade (Supporting Information Fig. S1); all comparisons are coloured by the habitat types of the OTU pair. In *Xantusia*, closely related species are geographically proximate. However, range overlap is uncommon between both closely related and distantly related OTU pairs.

for most of the divergence process. However, our D-statistic analyses identified multiple probable cases of introgression (Fig. 3; Supporting Information Fig. S6), suggesting that there was likely to be opportunity for sporadic hybridization among the species. In addition, we found some evidence of admixed individuals located near the parapatric borders between OTUs, including two probable cases of mtDNA introgression (Fig. S7) and three probable cases of nuclear admixture (Figs S8–S10). Our results identify a few OTU comparisons—i.e. *X. vigilis* KCAV and EM—where multiple approaches identified evidence for gene flow (Fig. 3), and we find multiple cases of gene flow between nonsister species with geographical distributions that are currently geographically disjunct—i.e. *X. gilberti* and *X. sanchezi*. However, although we might expect more gene flow between ecomorphs or between more closely related lineages, we find no such pattern (Fig. S11). Instead, gene flow in this group appears to be largely unpredictable and somewhat limited.

Under allopatric speciation, closely related, recently diverged species are not expected to overlap (Fitzpatrick *et al.* 2009, Rovito 2017), as seen in *Xantusia* (Fig. 6). As further evidence of allopatric speciation, breaks between *Xantusia* taxa align with known biogeographical breaks. For example, *X. bolsonae*, *X. extorris*, and *X. sanchezi* are found in continental Mexico while *X. gilberti* and *X. sherbrookei* are found in Baja California, *X. vigilis* EM is restricted to the Mojave Desert relative to the rest of its clade, which is found in nonarid environments, and *X.*

henshawi 2 and 1 are separated by the Elsinore Fault. However, even under allopatric speciation, we might expect that, with time, species will expand their ranges to come into secondary contact. Achieving secondary sympatry was a key part of the species cycle as envisioned by Mayr (1963), occurring only after species have accumulated sufficient reproductive barriers and ecological divergence to coexist stably. Yet, ranges in *Xantusia* are relatively stable. Although increased sampling at geographical gaps between species might change our inference, just three of 49 (6.1%) OTU comparisons showed evidence of significant overlap (measured here as > 20% overlap of the smaller range; Fig. 6). One of the few cases of overlap is between the rock ecomorph *X. bolsonae* and the plant ecomorph *X. extorris*, suggesting perhaps that niche divergence between these forms allows their stable coexistence. However, in the group as a whole, unlike what has been seen in other groups, neither time since split (Fitzpatrick *et al.* 2009, Rosser *et al.* 2015) nor ecological differentiation (Pigot and Tobias 2013, Glass *et al.* 2023) makes overlap more likely. Other taxon groups in which low overlap has been reported are gophers and plethodontid salamanders (Fitzpatrick *et al.* 2009, Rovito 2017), both of which—similar to *Xantusia*—are low-dispersing, secretive species. Possibly, these species are crawling towards bigger ranges very slowly and will require much more time before they achieve significant range overlap. Low range overlap might also reflect the effects of biogeographical history, as these ranges probably expanded and contracted in response to Pleistocene glacial cycles (Bezy *et al.* 2020). Alternatively, perhaps communities containing *Xantusia* are relatively stable, each hosting a single species of *Xantusia* that replaces congenerics in different geographical regions (see also *Ensatina* and *Batrachoseps*; Wake 2006).

Summarizing across these results, the story of speciation in *Xantusia* seems fairly straightforward. An ancestral population becomes two populations, perhaps due to vicariance or dispersal across a geographical barrier (like the San Jacinto and Elsinore faults that appear to split *X. henshawi* OTUs; Lovich 2001). As the populations diverge, these geographical barriers—combined with the low vagility of these species—maintain isolation with little to no opportunity for gene flow. In some cases, perhaps when forced into rock habitats during high wildfire regimes, the populations might become ecologically distinct. With enough time, the populations emerge as distinct species, with almost no build-up of *Xantusia* species diversity within an ecological community.

FUTURE DIRECTIONS

Through our review of the literature, and our own analysis of comparative speciation in *Xantusia*, we identified some open challenges and opportunities for our field that we detail below.

First, comparative studies of speciation require us to first ensure we have delimited units. For many taxa, existing taxonomies will suffice. Particularly for understudied taxa or taxa that exhibit minimal morphological divergence (Pfenninger and Schwenk 2007), existing taxonomies might be inaccurate. Our own study exemplifies this—the recognized taxonomy for *Xantusia* includes the species *X. vigilis*, a wide-ranging species that spans the southwestern USA and northern México. The new genome-scale data presented here confirm previous results (Sinclair *et al.* 2004, Leavitt *et al.* 2007) that this species is a paraphyletic assemblage of five OTUs (Fig. 2). Similarly, *X. henshawi* consists of three deeply divergent OTUs (Fig. 2). *Xantusia henshawi* and *X. vigilis sensu stricto* differ from their constituent OTUs across multiple axes, including levels of genetic diversity and geographical range size (Supporting Information Table S3). Thus, our taxonomic framework could have impacted subsequent inferences about the biology and history of a taxon. As such, before we could study speciation in *Xantusia*, we had to update the taxonomy. While inferring the revised taxonomy was relatively straightforward, we introduced circularity by using the same genetic data to both define our revised taxonomy and understand speciation dynamics. After all, key processes such as gene flow both influence species boundaries and our understanding of speciation dynamics [but see Smith and Carstens (2020) for a potential solution]. Further, while not an issue in this study, some comparative analyses—i.e. inferring speciation rates—require all units to be species-level, since the addition of population-level variation can bias results. Thus, how to effectively integrate species delimitation with studies of speciation remains an open challenge.

Once comparable units have been defined, comparative studies of speciation require comparative datasets. Collecting such datasets requires both broad-scale sampling across units and geography and identification of biologically relevant data that are homologous across scale. As we see in this study, biodiversity resources such as natural history museums can serve an indispensable role by allowing researchers to efficiently sample the geographical breadth of species (Nachman *et al.* 2023). As also seen in this study, the growth of high-throughput sequencing facilitates comparative work, as genomic data can make it easier and cheaper to collect homologous data across phylogenetic scales. However, other data remain challenging, expensive, and time-consuming to collect. For example, a limitation of our study is that we coded OTUs as either 'rock' or 'plant' ecomorphs based on literature surveys. However, species exhibit heterogeneity in ecomorph type (Bezy 2019)—e.g. some *X. vigilis* EM individuals have more rock-like morphology and habitat preference. Ideally, we would have captured this variance by measuring ecomorphology and habitat use to create a more continuous metric of habitat specialization; doing so would have required extensive museum and/or fieldwork, however. The difficulties of collecting comparative data can bias us towards studying well-resourced, tractable species and/or easy-to-measure traits such as body size and bioclimatic variables. Potential solutions include crowd-sourcing the digitization of trait data (Cooney *et al.* 2017a) and using machine learning on museum specimens or community science datasets (Hantak *et al.* 2022, Weeks *et al.* 2023). However, estimating RI—which in many ways has become synonymous with understanding speciation (Rabosky 2016)—has few shortcuts. Thus, how to effectively collect comparative datasets remains an open challenge.

Additionally, many of our current studies of comparative speciation—particularly those looking at pairwise divergence and those using comparative genomics—tend to focus on somewhat similar radiations. Popular systems for studying speciation include the *Timema* stick insects, *Heliconius* butterflies, and cichlid fish, all of which are relatively young, species-rich radiations consisting of parapatric or sympatric species exhibiting obvious ecomorphological differences. These aspects of these radiations make them excellent study systems. For example, because these lineages are young, we can capture the processes that initiate speciation. However, these same features might make these radiations outliers with respect to the rest of biodiversity. Some of the species in these radiations are probably ephemeral and bound for extinction (Rosenblum *et al.* 2012, Dynesius and Jansson 2014, Meier *et al.* 2023). These young radiations could thus bias our understanding of how speciation works, particularly if the factors that initiate speciation do not necessarily lead to persistence. Here, we see how speciation in *Xantusia* differs relative to these groups. *Xantusia* speciation appears to have occurred largely in physical and genetic isolation, with only rare occurrence of secondary sympatry. This contrasts with an emerging speciation model of frequent gene flow among interacting and often overlapping lineages (e.g. combinatorial speciation; Marques *et al.* 2019). Our work thus illustrates that considering a diversity of systems can help us understand the diversity of ways that speciation can occur. Thus, extending comparative approaches to the tree of life—not just recent, rapid radiations—is an opportunity for our field.

Finally, a benefit of comparative studies is that they allow us to integrate time and geography into studies of speciation. Speciation has an inherent spatial and temporal dimension, even if we typically can only study it from a few geographical locations and at a snapshot in time. However, all of the comparative approaches outlined here (Table 1) allow us to consider the effects of time, whether that is through its imprint on the historical demography of species (i.e. comparative phylogeography), through characterizing changing diversification dynamics (i.e. macroevolutionary studies), or through a deeper understanding of how patterns of divergence and RI change across lineage-pairs of different ages (i.e. pairwise estimates of divergence). Similarly, studies of comparative phylogeography, geographical overlap, and speciation across geographical regions allow us to integrate geography into our studies of speciation. As seen in *Xantusia*, geographical isolation and time are crucial ingredients in precipitating speciation, and comparative studies afford us the opportunity to understand how these factors might function in other radiations.

SUPPLEMENTARY DATA

Supplementary data are available at *Evolutionary Journal of the Linnean Society* online.

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

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DATA AVAILABILITY STATEMENT

Raw sequencing reads are available at NCBI BioProject PRJNA1122223. Scripts used in analysing and visualizing data are available at https://github.com/singhal/xantusia. Input and result files for population genetic, phylogenetic, and geographical analyses are available at https://github.com/singhal/xantusia.

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