



# Species delimitation in endangered groundwater salamanders: Implications for aquifer management and biodiversity conservation

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Contributed by David M. Hillis, November 20, 2018 (sent for review August 31, 2018; reviewed by Shawn R. Kuchta and C. Richard Tracy)

Groundwater-dependent species are among the least-known components of global biodiversity, as well as some of the most vulnerable because of rapid groundwater depletion at regional and global scales. The karstic Edwards–Trinity aquifer system of west-central Texas is one of the most species-rich groundwater systems in the world, represented by dozens of endemic groundwater-obligate species with narrow, naturally fragmented distributions. Here, we examine how geomorphological and hydrogeological processes have driven population divergence and speciation in a radiation of salamanders (*Eurycea*) endemic to the Edwards–Trinity system using phylogenetic and population genetic analysis of genome-wide DNA sequence data. Results revealed complex patterns of isolation and reconnection driven by surface and subsurface hydrology, resulting in both adaptive and nonadaptive population divergence and speciation. Our results uncover cryptic species diversity and refine the borders of several threatened and endangered species. The US Endangered Species Act has been used to bring state regulation to unrestricted groundwater withdrawals in the Edwards (Balcones Fault Zone) Aquifer, where listed species are found. However, the Trinity and Edwards–Trinity (Plateau) aquifers harbor additional species with similarly small ranges that currently receive no protection from regulatory programs designed to prevent groundwater depletion. Based on regional climate models that predict increased air temperature, together with hydrologic models that project decreased springflow, we conclude that Edwards–Trinity salamanders and other codistributed groundwater-dependent organisms are highly vulnerable to extinction within the next century.

endangered species | groundwater depletion | multispecies coalescent | phylogeography | species tree

Groundwater is the largest source of liquid freshwater on Earth, providing drinking water for billions of people (1) and irrigation for most of the world's agriculture (2), while at the same time sustaining aquatic and terrestrial ecosystem function (3–5). Although groundwater was once a seemingly limitless resource that made possible the “green revolution” and expanded the global food supply (6–8), groundwater depletion has become an intensifying global problem (9–13). Aquifer overdraft threatens water and food security (14–16), as well as natural capital and economic welfare (17, 18). Groundwater-dependent ecosystems provide essential services (5), including water purification (19), biodegradation (20, 21), and nutrient cycling (22, 23). The groundwater-obligate organisms (stygiobionts) underpinning these ecosystem services are among the least-known components of global biodiversity (24, 25), as well as some of the most vulnerable to extinction (see, e.g., refs. 26–28). They are particularly vulnerable because most have small distributions (25, 29) and are adapted to a narrow set of environmental conditions (3, 4, 30). The actual or functional extinction of narrowly endemic stygiobionts may reverberate through ecological networks via complex interactions among species (see, e.g., refs. 31 and 32). In the worst case, extinction may result in the impaired functioning of groundwater-dependent aquatic and terrestrial ecosystems, resulting in the deterioration of water quality (see, e.g., refs. 33–35).

The karstic Edwards–Trinity aquifer system (36) of west-central Texas (Fig. 1) is one of the most species-rich groundwater environments in the world, represented by dozens of endemic species with small, naturally fragmented distributions (37–43). Groundwater is especially valuable for municipal, irrigation, and recreational use in this semiarid region, owing to low rainfall, frequent droughts, and little permanent surface water (44, 45). Groundwater development since the 1950s (46), combined with more recent, rapid residential development (47), has resulted in intensive pumping, decreased well yields, local water table declines, and diminished baseflow to springs and streams (48–51). Aquifer drawdown and the resulting reduction or cessation of springflow has resulted in habitat loss and fragmentation for groundwater species, compounded by reduced water quality from urban development (52–55). As a result, 13 groundwater-dependent species endemic to the Edwards Aquifer are listed as threatened or endangered under the US Endangered Species Act (ESA) (56–58),

## Significance

Groundwater ecosystems deliver services that are vital to human well-being and environmental quality. Overexploitation of groundwater threatens biodiversity and ecosystem stability worldwide, underscoring the need to discover, describe, and sustain groundwater-dependent species before they are lost. Here we present a phylogenomic analysis of a salamander radiation endemic to the karstic Edwards–Trinity aquifer system of west-central Texas. This intensively used resource sustains narrow-range endemic groundwater species that are endangered with extinction due to aquifer overdraft. The federal Endangered Species Act has been used as a tool to bring regulation to groundwater withdrawal, but existing state law that treats groundwater as private property undermines conservation. Without ecologically relevant policies for sustainable use, unchecked groundwater depletion will likely result in species extinction and ecosystem degradation.

Author contributions: D.C.C. and D.M.H. designed research; T.J.D., A.M.W., and D.M.H. performed research; T.J.D., A.M.W., D.C.C., and D.M.H. analyzed data; and T.J.D. and D.M.H. wrote the paper.

Reviewers: S.R.K., Ohio University; and C.R.T., University of Nevada, Reno.

The authors declare no conflict of interest.

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Data deposition: Raw sequence reads have been deposited in the NCBI SRA database (accession no. PRJNA504898). Input files for all analyses have been deposited in the Dryad Digital Repository (doi: 10.5061/dryad.3k5c0sh).

See Commentary on page 2410.

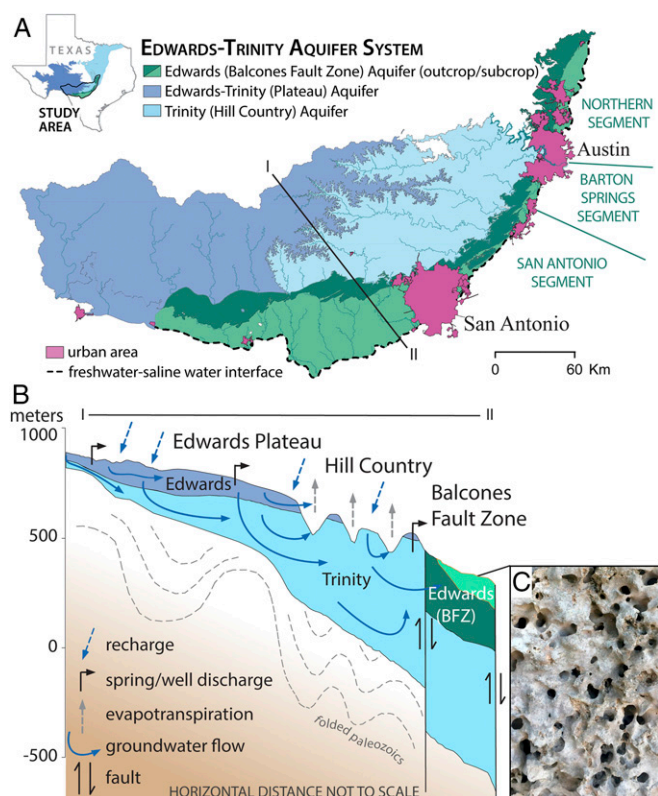
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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1815014116/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1815014116/-DCSupplemental).

Published online January 14, 2019.



**Fig. 1.** (A) Map of the study area illustrating the extent of the Edwards-Trinity aquifer system in west-central Texas. (B) Conceptual hydrogeologic cross section of the Edwards-Trinity (Plateau), Trinity (Hill Country portion), and Edwards (BFZ) aquifers. Younger Edwards Group rocks overlie Trinity Group rocks except in the Hill Country where the Edwards formation has been mostly eroded. Cross section modified from ref. 86. (C) Photo illustrating highly porous Edwards Group limestone.

including a narrowly endemic mosquitofish (the San Marcos *Gambusia georgei*) that has not been seen since 1983 and is presumed extinct (59).

Among the federally listed aquatic species, over half are plethodontid salamanders of the genus *Eurycea*, members of a radiation of about 14 species (60–62) found in oligotrophic environments of the Edwards Aquifer and its catchment area in the Edwards-Trinity (Plateau) and Trinity (Hill Country) aquifers. These salamanders occur in surface springs and spring-fed streams, as well as below ground in water-filled caves, solutionally enlarged conduits, and interstices of the porous limestone rock matrix. Some species are only temporary inhabitants of the subsurface aquifer, whereas others are obligately subterranean; a few have both surface (epigeal) and subsurface (hypogean) populations. Epigeal species are generally morphologically similar overall (63–65). Subterranean populations and species show phenotypic convergence (the independent evolution of similar traits) in their response to the challenges of life underground (62, 66–69). Previous genetic work using allozymes and mtDNA (60, 62) uncovered cryptic species (two or more morphologically similar species mistakenly classified as one; ref. 70) and revealed instances of shared ancestral polymorphism or introgressive hybridization that has made drawing species boundaries challenging. Species borders remain unclear for some taxa because many new populations have been discovered subsequent to the most recent taxonomic revision in 2000 (60). In some cases, newly discovered populations have been assigned to species only provisionally based on geographic proximity to known populations, without genetic evidence to support taxonomic designation (56). The accurate assignment of populations to species is essential in cases where the range size of a

taxon informs listing decisions about the vulnerability status of a species or the designation of critical habitat under the ESA (71, 72).

Because the species rank is the usual unit of currency for decision makers tasked with conservation prioritization, natural resource management, and environmental policy, accurately drawing species boundaries is critical for preventing the extinction of rare, cryptic species (see, e.g., ref. 73), spatial conservation prioritization (25), and ESA listing decisions (74). In this study, we use genome-wide DNA sequence data collected from over 300 individual Edwards-Trinity *Eurycea* salamanders to delimit evolutionary lineages at the species level and below. Our results uncover cryptic species and redraw the boundaries of some named species, including several that are listed under the ESA. These results have major implications for regional natural resource management and conservation because the ESA has been used as a tool to bring regulation to unrestricted groundwater withdrawal in the eastern half of the Edwards Aquifer where listed species are found (75–77). We also show that the Trinity and Edwards-Trinity (Plateau) aquifers harbor additional unnamed species that receive no protection, highlighting watersheds that deserve priority for biodiversity conservation.

### The Edwards-Trinity Aquifer System

Three major laterally adjacent and stratigraphically layered aquifers make up the Edwards-Trinity system, coincident with geographic subregions of the Edwards Plateau defined by distinct physiography, hydrology, and geology (Fig. 1A). From west to east, these aquifers are the Edwards-Trinity (Plateau) Aquifer underlying the Edwards Plateau and Trans-Pecos regions, the Trinity Aquifer of the Hill Country region, and the Edwards Aquifer of the Balcones Fault Zone (BFZ). The BFZ is a Miocene-age series of predominantly southeast-dipping normal faults trending southwest to northeast along the eastern edge of the plateau (78, 79) (Fig. 1A). The resulting displacement along the largest faults formed the Balcones Escarpment, a prominent topographic feature that affects regional weather and stream drainage patterns along the southern and eastern margins of the Edwards Plateau (80, 81). The Edwards-Trinity system is bounded along its southeastern edge by a well-defined freshwater-saline water transition that minimizes flow of freshwater from the Edwards Aquifer (82) (Fig. 1A).

The Edwards Aquifer is the most hydrologically active aquifer in the Edwards-Trinity system (36), discharging about 1.1 km<sup>3</sup> of water annually (83). The two southern segments of the Edwards (the Barton Springs and San Antonio segments) supply water to over 2.3 million people in the city of San Antonio and surrounding counties in south-central Texas and have been designated as sole-source aquifers for drinking water by the US Environmental Protection Agency (84). Aquifer thickness ranges from 60 to 180 m, with an average saturated thickness of 170 m in the southern portion (46). Regionally, the Edwards is subdivided by natural barriers into three segments. The San Antonio segment is separated from the Barton Springs segment by a groundwater divide near the city of Kyle in Hays County, and the Barton Springs segment is separated from the northern segment by the Colorado River (Fig. 1A). Locally, the southern segments of the Edwards Aquifer can be classified into three functionally distinct areas: the contributing zone, the recharge zone (Edwards outcrop), and the artesian or confined zone (Edwards subcrop). The contributing zone is the catchment area of the Edwards Plateau where precipitation infiltrates exposed Edwards and Trinity group rocks, migrating downward through the rock matrix by way of fractures and karst features such as sinkholes and directly recharging the water table (85) (Fig. 1B). Springs and seeps occur along the northern, eastern, and southern margins of the Edwards-Trinity (Plateau) Aquifer near the bases of the Edwards and Trinity groups where the water table intersects the land surface, discharging groundwater naturally to the surface under the force of gravity and providing base flow to streams that ultimately recharge the Edwards Aquifer downgradient (82, 86). Streams lose flow as they move across the exposed (unconfined)



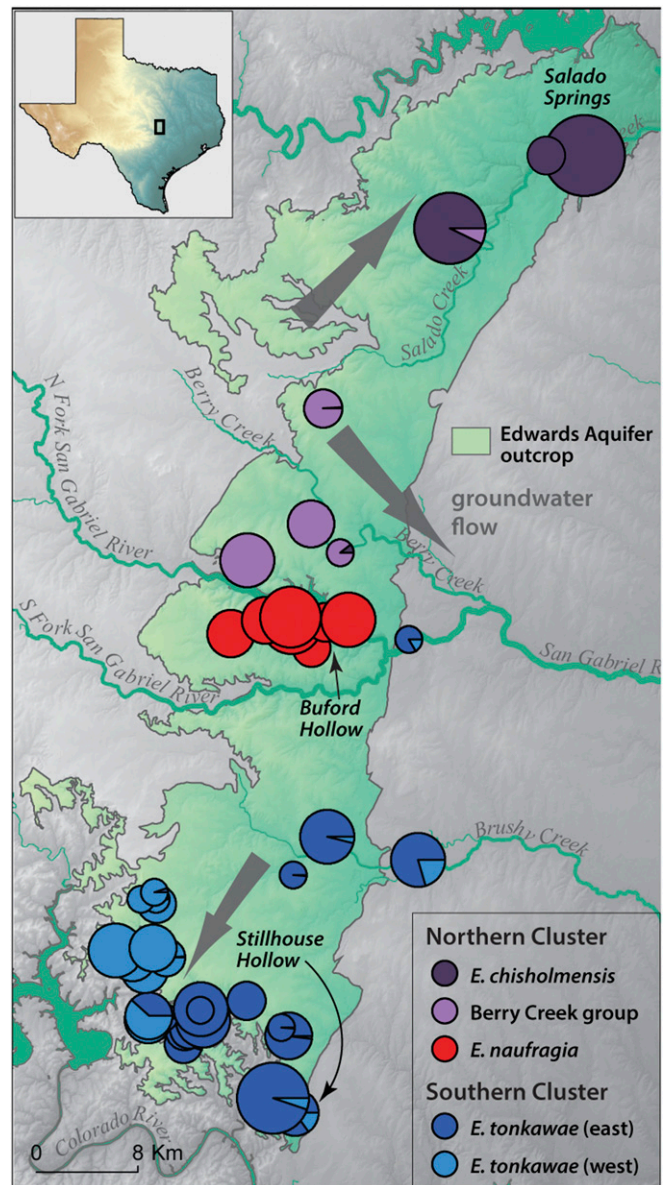
portion of the aquifer and recharge the water table through faults, fractures, and karst features. Unlike the southern segments of the Edwards, the northern segment lacks a distinct contributing zone; recharge occurs primarily through sinkholes on the aquifer outcrop (50, 87). In the confined zone, rock formations younger than Edwards cap the underlying aquifer, creating hydraulic pressure that forces water to the surface through the confining layer, creating natural discharge through artesian springs (Fig. 1B).

The unique geologic structure of the Edwards Aquifer controls the direction of groundwater movement, with permeable rock units horizontally adjacent to less-permeable units along fractures and faults that may act as either barriers or conduits to groundwater flow depending on the nature of the fault (88–90). Patterns of subsurface flow may be locally complex (91, 92), but in the southern segments water generally flows from areas of higher elevation in the southwest to discharge features in the northeast (36). Although the topographic boundaries between adjacent surface watersheds in the contributing zone of the Edwards Aquifer are well-defined, surface watershed boundaries may or may not be coincident with the extent of the underlying groundwater basins, especially where local geology is complex, for example in the northern segment of the Edwards (81, 93).

## Results

**Geographic Patterns of Population Structure.** Population structure of *Eurycea* in the Edwards–Trinity system is consistent with a hierarchical island model (94, 95), in which local subpopulations are clustered into neighborhoods within watersheds united by gene flow. Hierarchical *F* statistics calculated for a two-level hierarchy (subpopulation within watershed, and watershed within subbasin) revealed significant effects on overall differentiation at one or both levels (SI Appendix, Tables S1–S3). Bayesian assignment (96) revealed well-defined population structure, with individuals always being strongly assigned to a given population. Below, we detail geographic patterns of population structure by major geographic regions of the Edwards–Trinity system (excluding the subgenus *Typhlomolge*) as follows: (i) northern segment of the Edwards (BFZ) Aquifer (“northern region”), (ii) southeastern Edwards (BFZ) Aquifer and its contributing zone in the Hill Country portion of the Trinity Aquifer (“southeastern”), and (iii) southwestern Edwards (BFZ) Aquifer and its contributing zone in the Edwards–Trinity (Plateau) Aquifer (“southwestern”).

**Northern region.** Populations in the northern region have been classified as one of three distinct species (*Eurycea chisholmensis*, *Eurycea naufragia*, and *Eurycea tonkawae*) (60) that together comprise the highly divergent subgenus *Septentriomolge* (62). Hierarchical *F* statistics show a strong effect of subpopulation (within watershed) and watershed (within subbasin) on overall genetic structure (SI Appendix, Table S1). We analyzed population structure in a hierarchical fashion, beginning with all individuals and iteratively removing distinct clusters of individuals in subsequent analyses where we suspected additional subdivision exists. At the highest level (i.e., including all samples), individuals are strongly assigned to one of two population clusters (*K*) separated by the South Fork of the San Gabriel River (Fig. 2 and SI Appendix, Fig. S1). The northern cluster is further subdivided into three groups of populations, roughly corresponding to (from north to south) the Salado Creek, Berry Creek, and San Gabriel River (North and Middle forks) watersheds (Fig. 2 and SI Appendix, Fig. S2). The Salado Creek cluster includes the type locality for *E. chisholmensis* (Salado Springs) (60), whereas the North Fork of the San Gabriel River cluster includes the type locality for *E. naufragia* (Buford Hollow Spring) (60). Populations in the Berry Creek group have been assumed to represent *E. naufragia* based on geographic proximity to populations of that species, although most of these samples were collected subsequent to the most recent taxonomic revision in 2000 and had not been genotyped before this study. The southern cluster consists of populations classified as *E. tonkawae*, including the type locality at Stillhouse Hollow Springs (60) (Fig. 2). Within this cluster, indi-

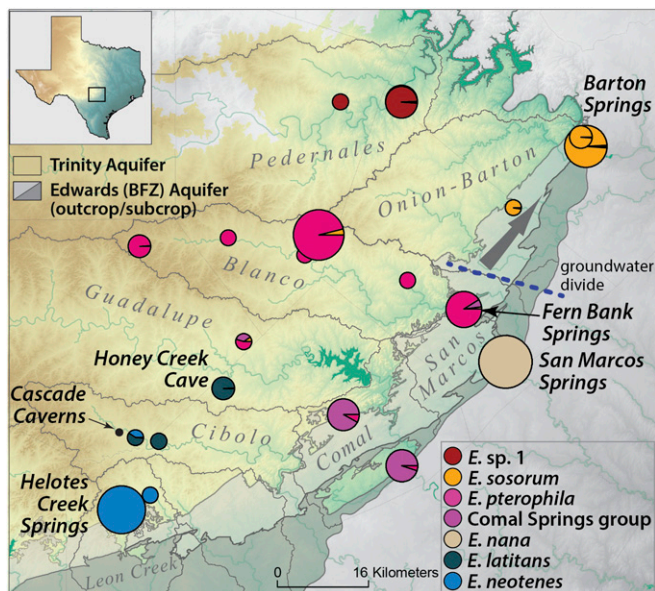


**Fig. 2.** Summary of hierarchical Structure analyses for the northern clade (subgenus *Septentriomolge*) showing the average individual membership coefficient (*Q*) for each sampling site. At the highest level of population structure ( $\text{Pr}(X|K = 2)$ ), individuals were assigned to northern and southern clusters separated by a hydrologic divide near the South Fork of the San Gabriel River. Each cluster is further subdivided, for a total of five major clusters shown here. Type localities for named species are shown. The size of each pie diagram is proportional to the number of sampled individuals per site ( $n = 1-9$ ). See SI Appendix, Figs. S1–S3 and S12A and Tables S6 and S7 for further detail.

viduals are strongly assigned to one of two distinct population groups (Fig. 2 and SI Appendix, Fig. S3). The eastern group is found primarily in the Bull Creek watershed, with peripheral subpopulations in the Brushy, Shoal, and Walnut Creek watersheds. The narrowly distributed western group is restricted to the Buttercup Creek and Cypress Creek subwatersheds. These eastern and western groups come into narrow contact along the divide separating the Cypress Creek and Bull Creek drainages, with individuals from two populations (SAS Canyon and Kretschmar Cave; SI Appendix, Fig. S12A) showing evidence of admixture.

**Southeastern region.** Within the southeastern region, seven clusters were inferred spanning (from north to south) the Middle





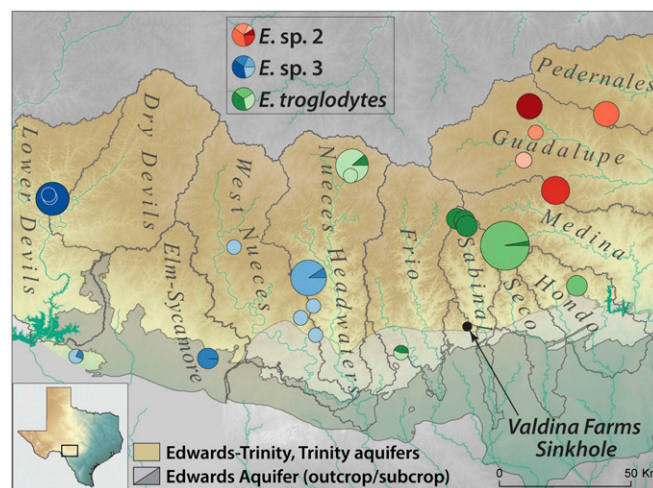
**Fig. 3.** Results of Structure analysis for the southeastern region (excluding the subgenus *Typhlomolge*) showing the average individual membership coefficient ( $Q$ ) for each sampling site for a representative run at  $K = 7$ . Type localities for named species are shown. Cascade Caverns (the type locality for *E. latitans*) was not sampled. The size of each pie diagram is proportional to the number of sampled individuals per site ( $n = 1-11$ ). See *SI Appendix, Figs. S4 and S12B and Tables S6 and S7* for further detail.

Colorado, Guadalupe, and San Antonio river basins (Fig. 3). Some of these clusters correspond to named species, while others represent intraspecific population groupings (*SI Appendix, Fig. S4*). Within the Middle Colorado, two clusters were inferred, one in the Pedernales subbasin corresponding to an undescribed species (hereafter, *E. sp. 1*) and one in the Barton Springs segment of the Edwards Aquifer (the Barton and Onion Creek watersheds), corresponding to *Eurycea sosorum* (60). Within the Guadalupe basin, *Eurycea nana*—the San Marcos Springs endemic—forms a distinct cluster. Remaining populations in the Guadalupe and San Antonio basins belong to the *Eurycea neotenes* species complex, a group of four named taxa (*Eurycea pterophila*, *E. neotenes*, *Eurycea latitans*, and *Eurycea tridentifera*) that exhibit extensive incomplete lineage sorting and/or introgressive hybridization based on mtDNA sequence data (65). These populations form five clusters that are consistent with named species or population groups found mostly in separate watersheds: Blanco River (*E. pterophila*), Comal River (*E. sp.*), Leon Creek (*E. neotenes*), and the Cibolo Creek (*E. latitans*) plus Honey Creek (*E. tridentifera*) catchments. Individuals from Honey Creek Cave (the type locality for *E. tridentifera*) are strongly assigned to a cluster with individuals from two populations in the Cibolo Creek Headwaters watershed. Although we did not sample from the type locality of *E. latitans* (Cascade Caverns), the single sample from nearby Pfeiffer's Water Cave shows evidence of admixture between the *E. latitans* cluster and the Leon Creek *E. neotenes* cluster (*SI Appendix, Fig. S4*).

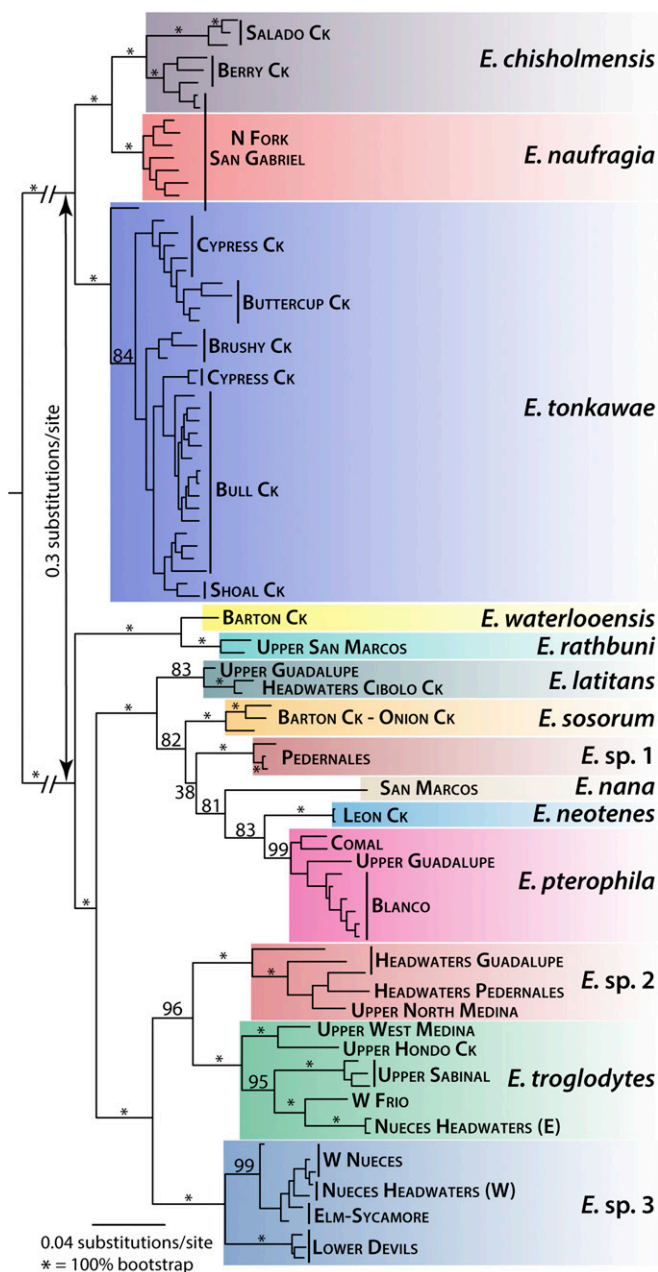
**Southwestern region.** Populations in the southwestern region belong to the *Eurycea troglodytes* species complex, a group of species found in the western Edwards and Edwards-Trinity (Plateau) aquifers (60). Sampled populations are strongly subdivided hierarchically by nested watershed (*SI Appendix, Table S3*). Three major, divergent clusters were inferred that correspond to distinct species (discussed below; see Fig. 4 and *SI Appendix, Figs. S5 and S12C*), and there is further subdivision within each species (*SI Appendix, Figs. S6, S8, and S9*). The easternmost cluster (hereafter, *E. sp. 2*) is found in the headwaters of the Guadalupe, upper North Medina, and Pedernales

River watersheds (Fig. 4 and *SI Appendix, Fig. S12C*). A second major cluster corresponding to *E. troglodytes* comprises populations found east of the Nueces River, in the eastern Nueces Headwaters, Frio, Hondo, and Upper West Medina watersheds (Fig. 4 and *SI Appendix, Figs. S8 and S12C*). The third, westernmost cluster (*E. sp. 3*) is found west of the Nueces River, in the Nueces Headwaters, West Nueces, Elm-Sycamore, and Lower Devils subbasins (Fig. 4 and *SI Appendix, Figs. S9 and S12C*).

**Population and Species Tree Estimation.** Maximum likelihood analysis (*SI Appendix, Methods*) and quartet inference under the multispecies coalescent model (97) of our largest assembly (74,955 SNPs) indicated similar relationships among populations; these two analyses (Fig. 5 and *SI Appendix, Fig. S10*) differ primarily in relationships within the southeastern region. These population trees are largely congruent with previous phylogenetic analyses of the group (60) but provide expanded coverage of the diversity and greater resolution of population assignment to species. The deepest division in the phylogeny separates northern and southern clades across the Colorado River and Mt. Bonnell fault of the BFZ. The northern clade (subgenus *Septentriomolge*) is represented by three named species (*E. chisholmensis*, *E. naufragia*, and *E. tonkawae*), two of which show clear evidence of additional geographic structuring across watersheds. Populations in the Berry Creek watershed are supported as the sister group of populations in the Salado Creek watershed (including topotypic *E. chisholmensis* samples) with strong support. Two geographic subgroups are also apparent within *E. tonkawae*, corresponding to eastern and western clusters of populations, but this division is not strongly supported. South of the Colorado River, the next deepest divergence separates the subterranean subgenus *Typhlomolge* (represented in our analysis by *Eurycea waterlooensis* and *Eurycea rathbuni*) from the remaining species. The remaining species (subgenus *Blepsimolge*) are split into strongly supported southeastern and southwestern clades. Population groupings within these clades are consistent with clusters recovered in Structure analyses. Within the southwestern clade, three major lineages were inferred with strong support. Within the southeastern clade, six major lineages were inferred, although relationships among



**Fig. 4.** Summary of hierarchical Structure analyses for the southwestern region showing the average individual membership coefficient ( $Q$ ) for each sampling site. The type locality for *E. troglodytes* (Valdina Farms Sinkhole) is shown but was not sampled. Three major clusters were inferred corresponding to distinct species, each of which is further subdivided. The size of each pie diagram is proportional to the number of sampled individuals per site ( $n = 1-11$ ). See *SI Appendix, Figs. S5-S9 and S12C and Tables S6 and S7* for further detail.



**Fig. 5.** Maximum likelihood tree of populations inferred from 74,955 SNPs. Branch labels are bootstrap support values from 1,000 replicates. See *SI Appendix, Fig. S11* for the same topology with subpopulation names shown. Watershed boundaries (*SI Appendix, Table S6*) are from the National Hydrography Dataset Plus (<https://www.epa.gov/waterdata/get-data>).

these lineages are not well-resolved, especially within the *E. neotenes* species complex.

#### Species Delimitation Under Bayesian Phylogenetics and Phylogeography.

Consistent with other studies that have used Bayesian Phylogenetics and Phylogeography (BPP) analysis (98, 99), the prior for  $\theta$  had considerable impact on species delimitation results (*SI Appendix, Table S4*). Across analyses under different priors, the ranking of the models usually remained the same, although the posterior probabilities for alternative delimitations varied. For the northern clade, we mapped individuals to five population groups (based on Structure results; see Fig. 2) representing potential species for BPP analysis: Salado Creek, Berry Creek, San Gabriel River, eastern *E.*

*tonkawae*, and western *E. tonkawae*. Across prior specifications, the posterior probability that these populations represent distinct species ranged from 0.71 to 1 (*SI Appendix, Table S4*). Berry Creek, Salado Creek, and San Gabriel River populations were usually recovered as distinct species with probability 1, whereas eastern and western *E. tonkawae* populations were usually grouped together. In the southeastern clade, individuals were mapped to nine population groups: Comal Springs, Cibolo Creek, San Marcos Springs, Leon Creek, Lower Blanco, Upper Blanco, Barton Springs segment, Pedernales River, and Honey Creek. Different specifications of the priors resulted in support for six to nine species. Blanco River populations were recovered as belonging to the same species, usually along with the Comal Springs and nearby Bear Creek Spring populations. Under a six-species model, the Cibolo Creek and Honey Creek population groups were grouped together as belonging to the same species. In the southwestern clade, all three population groups mapped as potential species (Fig. 4) were inferred to represent distinct species with probability 1 (*SI Appendix, Table S4*).

#### Discussion

##### Hydrogeology Drives Population Divergence in the Edwards–Trinity

*Eurycea*. Patterns of population divergence and speciation in the west-central Texas *Eurycea* have been driven in part by the complex surface and subsurface hydrogeology of the dynamic Edwards–Trinity system (64). At a regional scale, the boundaries of major clades coincide with major aquifer borders and distinct aquifer segments separated by hydrologic divides and the expression of major faults in the BFZ. Separation between the northern and southern clades across the Colorado River and Mt. Bonnell fault reflects deep evolutionary divergence, dating to at least the Middle Miocene (60). Population structure follows a hierarchical island model (95), where local subpopulations within nested catchment areas are united by gene flow. Patterns of species endemism range from single-site endemics to lineages that have widespread ranges spanning two or more subbasins. Species boundaries are frequently coincident with surface or subsurface hydrologic divides, consistent with a model of vicariant allopatric divergence and speciation. Alongside this nonadaptive population divergence are multiple instances of adaptive divergence between surface and subsurface intraspecific populations resulting in phenotypic convergence that has confounded previous species delimitation efforts based on morphology alone (69).

The intense faulting and fracturing in the BFZ and resulting structural controls on regional groundwater movement have driven population subdivision, and ultimately species formation, in this clade. In the northern region, subsurface groundwater divides separate distinct genetic clusters. The northernmost divide occurs between the Berry Creek and Salado Creek watersheds. North of this groundwater divide, groundwater follows northeast-trending faults and flows to the northeast, whereas south of this divide, groundwater in the Berry Creek drainage generally flows east (87) (Fig. 2). A second groundwater divide between Brushy Creek and the South Fork of the San Gabriel River similarly divides *E. naufragia* from *E. tonkawae* populations to the south on the Jollyville Plateau. South of this divide, groundwater flows southward, where it is captured by the deeply incised Colorado River along the southern margin of the Edwards Plateau (100). Faulting and an associated fault-bound relay ramp structure appear responsible for the east–west genetic subdivision within *E. tonkawae*, a pattern that is shared with other karst endemic species in the region (101). Cave populations in the Buttercup Creek karst (102) exhibit distinct troglomorphy and were hypothesized to represent a distinct species; these populations were only provisionally assigned to *E. tonkawae* (60). However, individuals from surface-dwelling populations in the Cypress Creek watershed are also strongly assigned to this same cluster, suggesting troglomorphic Buttercup Creek cave populations represent another instance of convergence (69). Dye tracing results have revealed subsurface connections between caves in the Buttercup Creek karst and



springs in the Cypress Creek watershed (103, 104) that may allow for gene flow between these separate drainages.

Local aquifer hydrodynamics are complex in the southeastern portion of the study area, where groundwater flow and recharge to the Edwards Aquifer is complicated by the structure of faults and the stratigraphy of rocks (105, 106). Water recharging the aquifer moves toward the northeast, along and parallel to northeast-trending faults, discharging at Comal, San Marcos, and Barton Springs. Surface and subsurface hydrologic divides serve as geographic barriers, but some are transient in nature. For example, a dynamic groundwater divide along Onion Creek near Kyle (Fig. 3) separates *E. sosorum* at the southern portion of its range from parapatric populations of *E. pterophila* in the Blanco River subbasin. North of this divide, groundwater in the Onion Creek watershed recharging the Edwards Aquifer usually flows northeast toward Barton Springs under average flow conditions (92). However, dye tracing has shown that during wet conditions groundwater can reverse direction, flowing up a structural dip toward San Marcos Springs instead of Barton Springs (92). This shifting groundwater divide may result in rare gene flow between watersheds, which may explain the pattern of mitochondrial DNA similarity between some individuals of *E. sosorum* from Barton Springs and *E. nana* at San Marcos Springs (65).

**Taxonomic Implications.** Our results have significant implications for the taxonomy of Edwards–Trinity *Eurycea* species, including ESA-listed and ESA-candidate species (*SI Appendix, Table S5*). In determining what constitutes a species, we view the various criteria used in delimiting species as contingent properties of metapopulation lineages that are evolving independently from other such lineages (107). Nonetheless, these contingent properties serve as important lines of evidence for assessing the evolutionary independence of lineages, including assessing their potential for gene flow or continued divergence. In determining which metapopulation lineages are evolving independently, it is useful to consider the evolutionary processes that result in lineage divergence, that is, natural selection, mutation, gene flow, and genetic drift (107). Of these evolutionary processes, we can say the most about gene flow based on available genetic data, although in some cases determining whether shared genetic variation is due to recent gene exchange or simply retained ancestral polymorphism following separation is challenging, particularly when populations have diverged only recently (108).

In the northern study area, our phylogenetic results show that all populations north of the San Gabriel River in and near the Berry Creek watershed are more closely related to *E. chisholmensis* than to *E. naufragia*, and so we assign these populations to *E. chisholmensis* (Fig. 6). These populations were initially assigned to *E. naufragia* (based on geographic proximity) when the three northern species were listed under the ESA in 2013 (56). Although the Berry Creek populations could be recognized as a new distinct species, rather than treated as a population segment of *E. chisholmensis*, there is evidence of gene flow or shared ancestral polymorphism between the Berry Creek and Salado Creek population groups (Fig. 2). Therefore, we consider these two population groups to be conspecific.

Our results also support assignment of the single salamander that is available from Georgetown Springs to *E. tonkawae* (*SI Appendix, Figs. S1 and S11*). However, this individual shows some indication of shared alleles with *E. naufragia* (*SI Appendix, Fig. S1*), suggesting possible past gene flow between *E. naufragia* and *E. tonkawae*. Nonetheless, this individual is not the result of a recent hybridization event, as it is genetically more similar to other individuals of *E. tonkawae* than any individuals of *E. naufragia* (*SI Appendix, Fig. S11*). This population may now be extinct, as we have been unable to locate any additional salamanders at Georgetown Springs since 1991.

Reassigning the Berry Creek and Georgetown Springs populations to other species significantly changes the known distribution of *E. naufragia* and necessitates revision of critical habitat designations under the ESA for *E. chisholmensis*, *E. naufragia*,

and *E. tonkawae*. Our results restrict the range of *E. naufragia* to springs south and east of Lake Georgetown in the North and Middle forks of the San Gabriel River watershed; all known populations of *Eurycea* north of Lake Georgetown are here included within *E. chisholmensis* (Fig. 6).

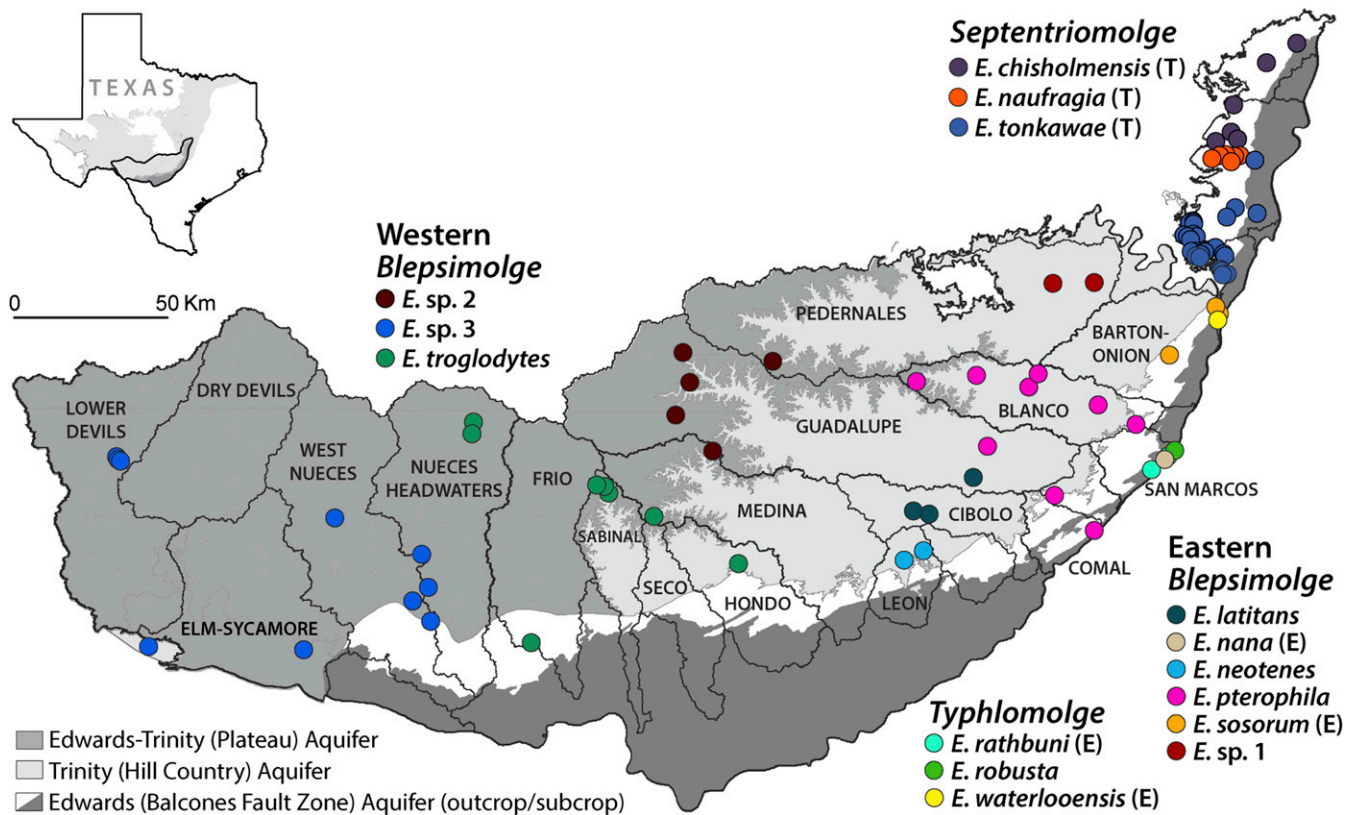
In the southeastern study area, our results suggest that species-level diversity has been overestimated within the *E. neotenes* species complex. Species in this group have been distinguished primarily by allozyme allele frequency differences (60). The population from Comal Springs has been suggested to represent a distinct species (60), and a petition for its listing has been filed under the ESA (109). However, individuals from Comal Springs and nearby Bear Creek Spring are genetically similar and share genetic variation with populations in the lower Blanco River and Guadalupe subbasins assigned to *E. pterophila* (see also ref. 110). We therefore recommend including both the Comal Springs and Bear Creek Spring populations within *E. pterophila* rather than recognizing them as a distinct, undescribed species (Fig. 6). Our results also show that *E. tridentifera* populations from the type locality at Honey Creek Cave group with *E. latitans* individuals from very near the type locality for that species at Cascade Caverns (Fig. 3), with little evidence to suggest that these two species are distinct. We therefore recommend that the name *E. tridentifera* (67) be subsumed under the older name *E. latitans* (111). Admixture between *E. latitans* and Leon Creek *E. neotenes* populations at Pfeiffer's Water Cave warrants further investigation with increased sampling (see also ref. 112).

In the southwestern study area, we recognize three species, two of them new. We restrict the name *E. troglodytes* to populations found east of the Nueces River, primarily in the Nueces Headwaters, Upper Frio, Hondo, and Medina subbasins (Fig. 6 and *SI Appendix, Table S6*). *Eurycea* sp. 2 appears to be restricted to springs in the headwaters of the Guadalupe, Pedernales, and North Prong of the Medina rivers. *Eurycea* sp. 3 is found west of the Nueces River, in the Nueces Headwaters, West Nueces, Elm-Sycamore, and Lower Devils subbasins (Fig. 6 and *SI Appendix, Table S6*).

It is important to note that, although the species rank is the usual unit of currency for decision makers tasked with conservation prioritization, a focus on the species level alone ignores geographic variation and population divergence within species such as *E. chisholmensis*, *E. tonkawae*, *E. pterophila*, *E. troglodytes*, and *E. sp. 3* (Figs. 2–4). Genetically divergent and geographically separated populations within these species (some of which have previously been considered to be full species) may warrant recognition as “distinct population segments” as defined under the ESA.

**Implications for Groundwater and Biodiversity Conservation.** The region bordering the eastern half of the Edwards Aquifer from San Antonio to the greater Austin area includes some of the fastest-growing counties and metropolitan areas in the United States (47, 113). Although the city of Austin does not rely directly on groundwater to meet its water demands, the city of San Antonio and many smaller municipalities rely largely or exclusively on groundwater from the Edwards Aquifer (114). Significant water table drawdown has already occurred over large portions of the study area, especially in the northern segment of the Edwards Aquifer (114, 115). From 1980 to 2000, groundwater pumping for municipal, rural domestic, and industrial use in the northern segment of the Edwards Aquifer nearly doubled (114). Between 1966 and 1990, over 50 m of drawdown occurred in some areas (116).

Major drawdown has also occurred over portions of the Trinity Aquifer. Groundwater yields from the Trinity Aquifer are about 250 times less than average yields in the adjacent Edwards Aquifer (117). Intensive pumping in the Trinity over the last several decades has resulted in water table declines, decreased well yields, and diminished baseflow to springs and streams (48, 118). The US Geological Survey, in cooperation with the US Fish and Wildlife Service, investigated the potential impacts of



**Fig. 6.** Map of sampled populations and species assignments inferred from results presented here, along with major watersheds in the study area. Species currently listed as Threatened (T) or Endangered (E) under the ESA are indicated.

groundwater pumping on known *Eurycea* spring localities located in the middle zone of the Trinity Aquifer (hereafter, Middle Trinity) (119). The Middle Trinity zone was chosen because it was the focus of a numerical groundwater availability model for the Trinity Aquifer (117). For Middle Trinity springs, projected water levels for both average recharge conditions and drought-of-record (DOR) conditions show drawdown at every spring. Under average recharge conditions projected for 2050, water table levels at 15 of 19 springs are predicted to decline by more than 3 m, whereas under DOR conditions water levels at 12 of 19 springs are projected to decline by more than 15 m (119).

Groundwater depletion, which results in diminished spring-flow and water table declines, is expected to have significant negative impacts on biodiversity and ecosystem functioning (30, 120, 121). This is especially true in shallow, dynamic karst aquifers (122) like those in the Edwards–Trinity system. For groundwater-obligate species such as *Eurycea* salamanders, declining water tables and reduced springflow causes habitat loss at the surface and subsurface, resulting in declines in individual abundance and eventually population extinction. Extirpations and functional extinctions (123) may then reverberate through ecological networks via complex interactions among species (see, e.g., refs. 31 and 32). In the worst case, population losses may result in the impaired functioning of groundwater-dependent ecosystems (see, e.g., refs. 33–35).

Effective conservation of groundwater-dependent biodiversity and ecosystem services faces a number of major challenges. The unique hydrogeologic properties of karstic aquifers that make them a valuable water source also make them especially vulnerable to contamination (124, 125). In west-central Texas, rapid population growth, increased water demands (126), and a warming climate with more frequent drought (127) are placing increased stress on limited water resources for human needs. During droughts, enforcement of the ESA requires that mini-

mum environmental flows be maintained to prevent the unlawful “take” of the Edwards Aquifer’s endangered species (128). However, state law in Texas treats surface water and groundwater as separate resources (despite their functional interdependence), with groundwater considered private property (129). Under this so-called rule of capture law, there is no enforceable legal mandate at the state or local level to maintain minimum aquifer levels (and hence springflow and stream baseflow) needed by endangered species (130). Without joint management of surface and subsurface waters as a single common-pool resource, the aquifers, springs, and streams of the Edwards–Trinity and the regional ecosystems they sustain will become increasingly threatened.

### Conclusions

Global change driven by human population growth (131), agricultural expansion (132), and climate change (133, 134) is steadily increasing the demand for freshwater, compounding the severe water scarcity already faced by an estimated 4 billion people (135). Groundwater is critically important for human health and ecosystem functioning in many semiarid regions because of low rainfall, frequent droughts, and little permanent surface water. The conservation of groundwater biodiversity should be a low-cost by-product of water conservation programs (29), but groundwater depletion increasingly threatens biodiversity as well as the stability of aquatic, terrestrial, and subsurface ecosystems. Because most groundwater species are difficult to monitor, abundance declines are likely to go unnoticed until after populations have already been lost. Regional climate models that predict increased air temperature (136, 137), together with hydrologic models that project decreased springflow (119, 138, 139), predict that Edwards–Trinity *Eurycea* salamander populations and other codistributed groundwater-dependent species are highly vulnerable to extinction within the next century.

## Methods

**Sampling.** We sampled 1–11 individuals from 99 spring or cave sites for a total of 303 samples representing all known species of Edwards–Trinity *Eurycea*, except *E. (Typhlomolge) robusta* (which is known from a single specimen collected in the 1950s) (*SI Appendix, Fig. S12 and Table S6*). Most specimens and tissues were collected previously and some have been analyzed for allozyme and/or mtDNA variation (60, 62, 65). Specimens, collecting data, and remaining tissue samples are housed in the Genetic Diversity Collection of the Biodiversity Center, Department of Integrative Biology, The University of Texas at Austin. Tissues and specimens were collected following The University of Texas at Austin IACUC protocol AUP-2018-00151.

**Restriction-Site-Associated DNA Sequencing.** Sequence data were collected using double-digest restriction-site-associated DNA sequencing (ddRADseq) following the protocol of Peterson et al. (140). Libraries were constructed and sequenced at the Genomic Sequencing and Analysis Facility, The University of Texas at Austin, on the Illumina HiSeq. 2500 platform (100-bp paired-end run). Additional details are provided in *SI Appendix, Methods*.

**RAD Data Assembly.** We used both the Stacks computational pipeline (141) and ipyrad (142) to filter and sort reads, identify loci de novo, and genotype individuals. Stacks was used to produce SNP-based datasets for phylogenetic and population structure inference using first reads only because they were of higher quality. Both first and second reads were used to identify full-sequence RAD loci with ipyrad for BPP analyses. Because the parameters used in de novo locus identification and genotyping may affect downstream analyses and resulting inference (143–145), we examined a range of values for parameters to optimize assembly in both pipelines. Additional details are provided in *SI Appendix, Methods*. RAD data assembly was performed on the Lonestar 5 high performance computing system at the Texas Advanced Computing Center, The University of Texas at Austin. Demultiplexed fastq files are available in the NCBI Sequence Read Archive (SRA) under study number PRJNA504898 (146). Raw data, barcodes, and assemblies are available in the Dryad data package associated with this article (147).

**Population Structure Analysis.** We assigned individuals to species based on multiple lines of evidence, including morphology, allozymes, mtDNA, and geographic distribution (60, 62, 65). For individuals from populations whose taxonomic assignment was uncertain, we used the Bayesian clustering method implemented in Structure (96) to assign individuals to species based on their multilocus genotype. Additional details are provided in *SI Appendix, Methods*. We also estimated hierarchical *F* statistics (subpopulation within watershed within subbasin) following ref. 148 and tested the statistical significance of these levels on population differentiation using a generalized likelihood-ratio test (149) implemented in the HierFstat package (150, 151) for *R* (152).

**Phylogenetic Inference.** We used the SVDquartets inference method of Chifman and Kubatko (97) implemented in PAUP\* v4 (153) to estimate the species tree and a population tree under the multispecies coalescent model using the 74,955-locus SNP dataset. This method assumes that any incongruence between an SNP genealogy and the species tree is due only to the stochastic coalescent process and uses the full data directly, incorporating both mutational and coalescent variance inherent in species-tree estimation (154). The observed site pattern distribution is used to infer the true split for quartets of taxa based on the best singular value decomposition (SVD) score (97), followed by the quartet assembly algorithm of Reaz et al. (155) for tree inference. We evaluated 100,000 random quartets, both with and without the Erik+2 normalization method of Fernández-Sánchez and Casanellas (156). Ambiguous changes were treated as missing. Nonparametric bootstrapping with 10,000 replicates was used to quantify uncertainty in the inferred splits and a 50% majority-rule consensus tree was constructed in PAUP\* and visualized in FigTree ([tree.bio.ed.ac.uk/software/figtree/](http://tree.bio.ed.ac.uk/software/figtree/)).

We also inferred the maximum likelihood phylogeny of the 74,955-locus dataset using RAxML v.8 (157) with a conditional likelihood method (158) that corrects for the exclusion of invariant sites (159) (-m ASC\_GTRGAMMA-asc-corr lewis). To find the best ML tree, the matrix was analyzed 1,000 times and the best tree was kept; 1,000 bootstrap replicates were performed to assess support, and we used the SumTrees program in the DendroPy Python library (160) to summarize bootstrap support values. RAxML analyses were performed on the CIPRES Science Gateway server v3.2 (161).

**BPP Species Delimitation.** We used the Bayesian Markov chain Monte Carlo method of Yang and Rannala implemented in the program BPP v3.3a (162–165) to generate the posterior probabilities of different species delimitations based on support for recent coalescence. This method is a full likelihood-based implementation of the multispecies coalescent model, accounting for the species phylogeny while accommodating uncertainty due to unknown gene trees (topologies and branch lengths) at individual loci, and incomplete lineage sorting. Assumptions include no recombination within a locus and free recombination between loci, neutral evolution at a constant rate at each locus under the JC69 model of nucleotide substitution, and no gene flow between species (165). The data for these analyses are full sequences (including invariant sites) that are 108 bp in length. Additional details are provided in *SI Appendix, Methods*.

**ACKNOWLEDGMENTS.** We thank Craig Farquhar of the Texas Parks and Wildlife Department for assistance; Nathan Bendik, Paul Chippindale, Andy Gluesenkamp, John Karges, Lisa O'Donnell, Chad Norris, Colin Peden, and the late Andy Price for tissue samples; Deren Eaton and Isaac Overcast for assistance and advice with their program ipyrad; and Joe Allen, Richard Evans, Joe Garcia, Doug James, Antia Lamas-Linares, Jawon Song, and Virginia Truehart for assistance with Texas Advanced Computing Center resources. This work was funded by the US Fish and Wildlife Service, Section 6 Grant TX E158-R-1, through Texas Parks and Wildlife Department Contract 443022.

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