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Phylogenetic relationships of the North American catfishes (Ictaluridae, Siluriformes): Investigating the origins and parallel evolution of the troglobitic species

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ABSTRACT

Insular habitats have played an important role in developing evolutionary theory, including natural selection and island biogeography. Caves are insular habitats that place extreme selective pressures on organisms due to the absence of light and food scarcity. Therefore, cave organisms present an excellent opportunity for studying colonization and speciation in response to the unique abiotic conditions that require extreme adaptations. One vertebrate family, the North American catfishes (Ictaluridae), includes four troglobitic species that inhabit the karst region bordering the western Gulf of Mexico. The phylogenetic relationships of these species have been contentious, and conflicting hypotheses have been proposed to explain their origins. The purpose of our study was to construct a time-calibrated phylogeny of Ictaluridae using first-occurrence fossil data and the largest molecular dataset on the group to date. We test the hypothesis that troglobitic ictalurids have evolved in parallel, thus resulting from repeated cave colonization events. We found that *Prietella lundbergi* is sister to surface-dwelling *Ictalurus* and that *Prietella phreatophila* + *Trogloglanis pattersoni* are sister to surface-dwelling *Ameiurus*, suggesting that ictalurids colonized subterranean habitats at least twice in evolutionary history. The sister relationship between *Prietella phreatophila* and *Trogloglanis pattersoni* may indicate that these two species diverged from a common ancestor following a subterranean dispersal event between Texas and Coahuila aquifers. We recovered *Prietella* as a polyphyletic genus and recommend *P. lundbergi* be removed from this genus. With respect to *Ameiurus*, we found evidence for a potentially undescribed species sister to *A. platycephalus*, which warrants further investigation of Atlantic and Gulf slope *Ameiurus* species. In *Ictalurus*, we identified shallow divergence between *I. dugesii* and *I. ochoterenai*, *I. australis* and *I. mexicanus*, and *I. furcatus* and *I. meridionalis*, indicating a need to reexamine the validity of each species. Lastly, we propose minor revisions to the intrageneric classification of *Noturus* including the restriction of subgenus *Schilbeodes* to *N. gyrinus* (type species), *N. lachneri*, *N. leptacanthus*, and *N. nocturnus*.

1. Introduction

Insular habitats have played an important role in advancing our understanding of evolutionary processes (Darwin, 1859; Brown, 1978; Schluter, 1988; Losos & Ricklefs, 2009; Lescak et al., 2015). Insular habitats are suitable for the persistence of adapted organisms, but are surrounded by habitat that is unsuitable for those organisms (Brown, 1978; Acosta, 1999). Using oceanic islands as an example of insular habitats, MacArthur and Wilson (1967) developed one of the most influential theories in evolutionary biology, *The Theory of Island Biogeography* (Whittaker et al., 2008; Patiño et al., 2017). They proposed that species richness on oceanic islands is a function of island area and distance from the continent through their effects on rates of immigration and extinction (MacArthur & Wilson, 1963; MacArthur & Wilson, 1967). Owing to the central role of islands in the development of the theory of island biogeography, insular habitats have proven invaluable for better

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understanding how processes including colonization, habitat complexity, habitat connectivity, and extinction influence species diversity (MacArthur & Wilson, 1967; Whittaker et al., 2008).

Islands are not the only example of insular habitats. Mountain peaks, ponds, lakes, caves, and natural habitats fragmented by anthropogenic activities may also function as insular habitats (Brown, 1971; Whittaker et al., 2008). Caves differ markedly from other insular habitats because their abiotic conditions are unique, relatively uniform among cave systems, and temporally stable (Culver, 1970; Salin et al., 2010; Ferreira & Pellegrini, 2019). For example, cave temperature, light availability, and humidity exhibit low variation in comparison with surface habitats (Salin et al., 2010; Ferreira & Pellegrini, 2019). Similar abiotic conditions in different cave systems have resulted in convergent evolution of troglobitic species spanning the kingdom Animalia (Barr & Holsinger, 1985; Mammola & Isaia, 2017). These animals possess similar traits, such as unpigmented skin and non-functional eyes (Christman et al., 2005; Culver & Pipan, 2015). Dispersal into caves and selection for traits required to live in these extreme environments are driving forces of speciation, ecologically isolating these populations from their surface-dwelling relatives (Christiansen, 1961; Barr & Holsinger, 1985; Strecker et al., 2012). Owing to their insular nature and to the relative uniformity of selective pressures, caves provide an excellent opportunity to improve our understanding of colonization, convergent and parallel evolution, and speciation (Culver, 1970; Culver, 1976; Culver & Pipan, 2010).

One vertebrate family containing several troglobitic species is the North American catfishes, Ictaluridae (Lundberg, 1970; Lundberg, 1992; Walsh & Gilbert, 1995; Burr et al., 2020). This monophyletic family occurs from Southern Canada to Guatemala (Fig. 1), inhabiting a wide diversity of lentic and lotic habitats, such as creek riffles, large river channels, lakes, and subterranean pools (Nelson, 2006; Arce H. et al., 2016; Burr et al., 2020). Fossils extend the historical distribution of ictalurids to the Pacific Northwest of the United States (Lundberg, 1992). Ictaluridae comprises seven genera, including four surface genera (*Ameiurus*, *Ictalurus*, *Noturus*, and *Pylodictis*) and three cave genera (*Prietella*, *Satan*, and *Trogloglanis*) (Fig. 1; Walsh & Gilbert, 1995; Wilcox et al., 2004). There are currently 50 recognized extant species in the family, of which four are troglobitic: *Prietella lundbergi*, *Prietella phreatophila*, *Satan eurystomus*, and *Trogloglanis pattersoni* (Wilcox et al., 2004; Nelson, 2006; Arce H. et al., 2016; Burr et al., 2020). These four troglobitic species exhibit similar morphological adaptations to subterranean life observed in other aquatic cave fauna, such as non-functional eyes, achromatism, reduced swim bladder size, and fragmented lateral-line systems (Walsh & Gilbert, 1995; Arce H. et al., 2016).

Troglobitic ictalurids are found in the karst region surrounding the Gulf of Mexico, from southern Texas, USA, to southern Tamaulipas, Mexico (Fig. 1; Wilcox et al., 2004; Sharp et al., 2019; Burr et al., 2020). *Satan eurystomus* and *Trogloglanis pattersoni* co-occur in the San Antonio Pool of the Edwards Aquifer to depths of at least 300 m below sea level (Langecker & Longley, 1993; Walsh & Gilbert, 1995). *Prietella phreatophila* occurs in underground streams in deep caves in northern Coahuila, Mexico, as well as in two caves in Amistad National Recreation Area north of the Rio Grande (Walsh & Gilbert, 1995; Hendrickson et al., 2001; Hendrickson et al., 2017; Krejca & Reddell, 2019; GBIF.org, 2022). There are hydrological connections between some of these localities, but the extent of connectivity between collection sites is still largely unknown (Hendrickson et al., 2001). *Prietella lundbergi* occurs further south than other troglobitic ictalurids, specifically in two subterranean springs in the Tamesí River drainage in southern Tamaulipas (Walsh & Gilbert, 1995; Hendrickson et al., 2001). The cave systems that *P. phreatophila* and *P. lundbergi* inhabit are separated by approximately 600 km and extensive mountain ranges (Hendrickson et al., 2001; Wilcox et al., 2004). These cave systems are in two karst regions, the Coahuila and Sierra Madre Oriental karsts, respectively (Espinasa-Pereña,

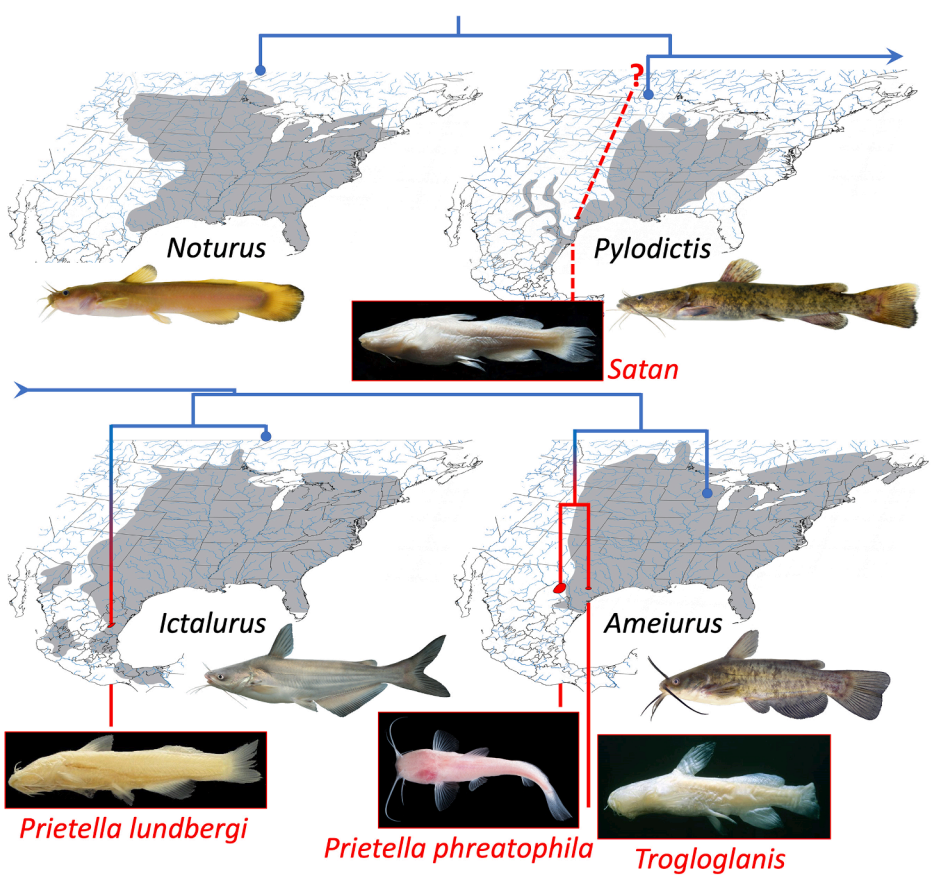

Fig. 1. Phylogeographic sketch of extant Ictaluridae based on relationships supported in this study (solid lines) or inferred from previous ones (dashed line). Branch lengths proportional to those in Fig. 2; circles denote common ancestor of respective genus. Distribution maps of epigean genera (gray) and hypogean species (red) derived from Burr et al. (2020). Photos by D.A. Hendrickson (*P. lundbergi*), J. Krejca, Zara Environmental LLC. (*P. phreatophila*), M.H. Sabaj (*Noturus*, *Pylodictis*), G.W. Sneegas (*Satan*, *Trogloglanis*) and M.R. Thomas (*Ameiurus*, *Ictalurus*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2007). As such, it is highly unlikely that there are hydrological connections between them (Hendrickson et al., 2001; Wilcox et al., 2004). *Prietella phreatophila*, *S. eurystomus* and *T. pattersoni*, however, all occur in aquifers developed in the Edwards Formation that underlies much of southwestern Texas and northern Coahuila (Sanchez et al., 2016; Sanchez et al., 2018a). That parts of this broad complex of variably interconnected aquifers, all recharged by runoff from the Edwards Plateau, are international is now well demonstrated, as are subterranean aquifer interconnections that extend far west of the ranges of *S. eurystomus* and *T. pattersoni* (under San Antonio) to very near the Texas *P. phreatophila* localities (Boghici et al., 2004; Sanchez et al., 2016; Lundberg et al., 2017; Sanchez et al., 2018a; Sanchez et al., 2018b).

The phylogenetic positions of the four troglobitic ictalurids have always been contentious. Despite their convergent cave adaptations, *Prietella*, *Satan*, and *Trogloglanis* share traits with surface-dwelling genera (Hubbs & Bailey, 1947; Langecker & Longley, 1993; Wilcox et al., 2004). This caused confusion in early morphological classifications, which disagree with respect to intergeneric relationships. *Prietella* was often considered to be most closely related to *Noturus* (Taylor, 1969; Lundberg, 1970; Lundberg, 1982; Walsh & Gilbert, 1995); however, Suttkus (1961) proposed that *Ictalurus* + *Ameiurus* were close relatives of *Prietella*. The phylogenetic position of *Trogloglanis* varied widely between studies. Upon its description, Eigenmann (1919) proposed *T. pattersoni* shared a common ancestor with *Noturus*. As additional morphological and anatomical data were collected, the phylogenetic position of *Trogloglanis* changed, being grouped with *Ameiurus* (Hubbs & Bailey, 1947) or *Ictalurus* (Taylor, 1969), placed sister to all ictalurids except *Ictalurus* (Lundberg, 1970; Lundberg, 1982), or placement uncertain (Lundberg, 1970). Unlike *Prietella* and *Trogloglanis*, morphological studies consistently agreed that *S. eurystomus* and *Pylodictis olivaris* formed a sister pair (Hubbs & Bailey, 1947; Taylor, 1969; Lundberg, 1982; Lundberg, 1992; Lundberg et al., 2017) due to several shared internal and external traits, such as a flattened head, flaring adipose fin, and a broadly-forked mesethmoid (Hubbs & Bailey, 1947; Suttkus, 1961; Taylor, 1969; Lundberg, 1970; Lundberg, 1982; Lundberg et al., 2017).

More recent studies have included molecular data for phylogenetic analyses of Ictaluridae (e.g., Hardman & Hardman, 2008); however, to date, only *P. phreatophila* and *P. lundbergi* have been included in molecular phylogenies (Wilcox et al., 2004; Egge & Simons, 2009; Arce H. et al., 2016). Similar to morphological studies, molecular analyses disagree on the phylogenetic position of both *Prietella* species. Maximum Parsimony (MP) analyses of molecular sequence data support *Prietella* as monophyletic (Wilcox et al., 2004; Arce H. et al., 2016), whereas Bayesian Inference (BI) and Maximum Likelihood (ML) analyses place each species of *Prietella* sister to a surface-dwelling genus (Wilcox et al., 2004). Arce H. et al. (2016) combined fossil, morphological, and genetic data (one nuclear and four mitochondrial genes). This time-calibrated MP phylogeny included representatives of all extant and extinct ictalurids, except for three *Ictalurus* species (Arce H. et al., 2016). Genetic data were analyzed for the two *Prietella* species, but *Satan eurystomus* and *Trogloglanis pattersoni* were included in the phylogeny based only on morphological characters. In contrast to previous phylogenies, Arce H. et al. (2016) found that the four cave species comprise a monophyletic Troglobites clade, sister to all other ictalurids; this placement was further supported by Lundberg et al. (2017) with morphological characters. This unexpected result implies that these four species descended from a common ancestor, despite their allopatric distribution (*Satan* and *Trogloglanis* excepted). Arce H. et al. (2016) mention that the putative dispersal events require further investigation, given that hydrological connections are largely unknown between cave locations.

Due to the phylogenetic contradictions concerning troglobitic ictalurids in previous studies, we aimed to determine the evolutionary origins of these species. We elucidated the evolutionary history of Ictaluridae by creating a species-level molecular phylogeny using the largest molecular dataset of any study to date, as well as the first inclusion of genetic data for *Trogloglanis pattersoni*. We then time-calibrated the phylogeny using the earliest fossil occurrence data of ictalurid and siluriform outgroups to determine when cave colonization events may have occurred. Using our time-calibrated phylogeny, we tested the hypothesis that ictalurid cave species have evolved in parallel from repeated invasions of subterranean habitats. We predicted that the troglobitic ictalurids are not monophyletic and are each sister to surface-dwelling taxa, as observed in other troglobitic fishes (Wilkens, 2001). Given the distance and geological barriers between their known locations, we also predicted that *P. phreatophila* is sister to *Ameiurus* and *P. lundbergi* is sister to *Ictalurus*, as observed in previous molecular-only phylogenies (Wilcox et al., 2004; Egge & Simons, 2009).

2. Materials and methods

2.1. Taxon sampling and gene selection

To construct our species-level phylogeny of Ictaluridae, we used muscle and fin tissue samples from museum-deposited voucher specimens and field-collected specimens. When possible, we included two voucher specimens per ingroup species from different localities. We obtained all nominal extant species except *Satan eurystomus*. *Satan eurystomus* is a rare species that has not been collected since 1984 and no useable tissue samples are available from formalin-fixed specimens (Wilcox et al., 2004; Lundberg et al., 2017). We selected 17 outgroup species from closely-related families within Siluriformes (Teleostei, Actinopterygii) to root the tree, representing Austroglanididae, Bagridae, Clariidae, Claroteidae, Cranoglanididae, Mochokidae, Pangasiidae, and Sisoridae (Betancur-R et al., 2017; Schedel et al., 2022). Lastly, we included eight potentially undescribed ictalurid species in our phylogeny. We used at total of 118 specimens (Table S1).

We selected 9 protein-coding genes to construct our phylogeny, including two mitochondrial genes and seven nuclear genes. We also downloaded mitochondrial sequences from GenBank (Clark et al., 2016) composed of 12S ribosomal RNA, tRNA-Val, and 16S ribosomal RNA for as many available catfish species as possible. These genes were chosen based on three criteria: (1) inclusion of a combination of quickly-evolving and slowly-evolving genes to provide phylogenetic resolution for both interspecific and intergeneric relationships (Lovejoy, 2000; Le et al., 2006), (2) single-copy genes to prevent biases associated with sequencing gene paralogs (Lovejoy & Collette, 2001; Li et al., 2007; Chen et al., 2008; Hughes et al., 2018), and (3) inclusion of previously published sequences from rare species when genetic tissue samples were unavailable. The two mitochondrial genes we selected for phylogenetic analyses were cytochrome *c* oxidase subunit 1 (*co1*) and *cyt b*. The seven nuclear genes we selected for phylogenetic analyses were early growth response protein 1 (*egr1*), ectoderm-neural cortex protein 1 (*enc1*), glycosyltransferase (*glyt*), recombination activating gene 1 (*rag1*), recombination activating gene 2 (*rag2*), rhodopsin (*rh1*), and zinc finger protein of cerebellum 1 (*zic1*).

2.2. DNA sequencing and alignment

We extracted DNA from muscle and fin tissues stored in 95% ethanol at −20 °C using a homemade kit for animal extractions following a protocol adapted from Ivanova et al. (2006). Each extraction was amplified for nine of the selected genes (as described above) using the polymerase chain reaction (PCR). We used previously published primers to amplify *co1* (Ward et al., 2009) and *cyt b* (Palumbi et al., 1991), and designed primers to amplify each nuclear gene. For *co1* and *cyt b*, we used the same primers for amplification and sequencing. For all nuclear genes, we designed internal primers for sequencing to avoid non-specific PCR products amplification (Table S2).

To amplify *cyt b*, *enc1*, *rag2*, and *zic1*, we used the following PCR recipe for each reaction: 1X Dream buffer containing 2 mM $MgCl_2$, 0.2 mM of deoxynucleotides (dNTPs), 0.25 μM of forward and reverse

primers, 0.75 U of Dream *Taq*, ca. 20–30 ng of template DNA, and nuclease-free water to adjust to the final reaction volume of 15 μL. We used a Mastercycler pro S (Eppendorf Canada) to amplify our PCR products with the following heat cycling conditions: initial heating to 95 °C for 3 min, 35 cycles of denaturation (95 °C for 30 s), primer annealing (55 °C for 30 s), and extension (72 °C for 1 min and 30 s) phases, and a final extension phase at 72 °C for 10 min. For *co1*, *egr1*, *glyt*, *rag1*, and *rh1*, we used the following PCR recipe: 1X Q5 buffer containing 2 mM $MgCl_2$, 0.2 mM of dNTPs, 0.5 μM of forward and reverse primers, 0.45 μL of dimethyl sulfoxide (DMSO), 0.3 U of Q5 polymerase, ca. 20–30 ng of template DNA, and nuclease-free water to adjust to the final reaction volume of 15 μL. Cycling conditions for amplification were as follows: initial heating to 98 °C for 30 s, 34 cycles of denaturation (98 °C for 10 s), primer annealing (56 °C for 30 s), and extension (72 °C for 30 s) phases, and a final extension phase at 72 °C for 5 min.

PCR products were visualized using gel electrophoresis and products were subsequently diluted with a volume of nuclease-free water dependent on the strength of the band. Strong bands were diluted with 60 μL of water, medium bands were diluted with 30 μL of water, and weak bands were diluted with 15 μL of water. Diluted products were then used in sequencing reactions, each containing 0.9X ABI buffer, 0.5 μM of primer, 0.5 μL of BigDye containing dideoxynucleotides (ddNTPs), 1 μL of diluted PCR products, and nuclease-free water to adjust to the final reaction volume of 10 μL. Sequencing reactions were then run through the Mastercycler pro S using the following cycling conditions: initial heating to 95 °C for 3 min, 25 cycles of denaturation (96 °C for 30 s), primer annealing (50 °C for 20 s), and extension (60 °C for 4 min) phases. Sequencing reactions were then purified using an EDTA-NaOH-ethanol precipitation protocol provided by the manufacturer. DNA pellets were resuspended using HIDI formamide and sequenced using a 3500 xL Genetic Analyzer (ThermoFisher Scientific). GenBank accession numbers for sequenced genes are provided in Table S1. Each individual gene was aligned with the Clustal Omega plugin 1.2.2 (Sievers et al., 2011) in Geneious Prime 2022.0.2 (Geneious Prime, 2022). Gene alignments were then concatenated using SequenceMatrix 1.8 (Vaidya et al., 2011).

2.3. Phylogenetic analyses

We constructed a species-level phylogeny of Ictaluridae using a ML analysis. We first partitioned the concatenated alignment by gene and codon position, resulting in 30 partitions. Using PartitionFinder2 2.1.1 (Lanfear et al., 2017) on the CIPRES Science Gateway (Miller et al., 2011), we selected the best substitution model for each partition with the Corrected Akaike Information Criterion (AICc). Unlinked substitution models that best fit each partition were selected from those available for IQ-TREE (Nguyen et al., 2015; Table S3). Using the substitution models of best fit, we then performed a ML analysis to construct our phylogeny using the IQ-TREE web server (Trifinopoulos et al., 2016). The analysis was run for 100 likelihood searches and branch support was calculated using the ultrafast bootstrap (BS) approximation for 1000 replicates (Minh et al., 2013). Finally, we used FigTree 1.4.4 to visualize the output tree file (Rambaut, 2009).

2.4. Fossil-based divergence-time estimation of Ictaluridae

To construct a time-calibrated phylogeny of Ictaluridae, we performed a BI analysis using the CladeAge package (Matschiner et al., 2017) in BEAST2 2.6.3 (Bouckaert et al., 2019). First, we created the necessary XML file in BEAUti2 2.6.3 (Bouckaert et al., 2019) to time-calibrate our phylogeny using fossil-record information available for ingroup ictalurids and outgroup siluriforms. The concatenated gene matrix was partitioned by gene and codon position, resulting in 30 partitions. The substitution models that best fit each partition were determined with the bModelTest package (Bouckaert & Drummond, 2017) in BEAST2 using a reversible jump Markov chain Monte Carlo (MCMC). For the clock models, we selected an uncorrelated lognormal relaxed molecular clock model (Drummond et al., 2006) for the mitochondrial genes and for the nuclear genes separately. We used a birth–death process model for the tree prior (Kendall, 1948), specifying teleost-specific net diversification rate parameters (λ-μ; 0.041–0.081), turnover rate parameters ($\mu\lambda^{-1}$; 0.0011–0.37), and fossil sampling rate parameters (ψ; 0.0066–0.01806) based on previous studies (Foote & Miller, 2007; Santini et al., 2009; Matschiner et al., 2017); our ML phylogeny was used as a starting tree prior for the analysis. CladeAge accounts for clade age estimation uncertainty by inferring the optimal shape of calibration densities of each clade, combining sampling rates, diversification rates, and first-occurrence fossil ages (Matschiner et al., 2017).

We included 17 first-occurrence catfish fossils to time-calibrate clades using their minimum and maximum age estimations. Ictalurid fossils from Arce H. et al. (2016) were used to calibrate ingroup clades and first-occurrence outgroup fossils were selected using Database of Vertebrates: Fossil Fishes, Amphibians, Reptiles, and Birds database (Böhme & Ilg, 2003), the Paleobiology Database (Paleobiology Database, 2018), and original species descriptions. We calibrated *Ameiurus* using the oldest known fossil species belonging to the genus, †*Ameiurus pectinatus* from the Late Eocene, 34–34.2 million years ago (mya) (Lundberg, 1975; Lundberg, 1992; Hardman & Hardman, 2008; Arce H. et al., 2016). This fossil lineage is considered a member of *Ameiurus* given its synapomorphies, including an anteroventral crest of the dentary, broad snout, and broad premaxillae (Lundberg, 1975). We calibrated *Ictalurus* using the oldest fossil species, †*Ictalurus rhaeas* from putatively Late Eocene deposits 30–37 mya of the Cypress Hills Formation (Cope, 1891; Lundberg, 1975; Hardman & Hardman, 2008; Arce H. et al., 2016). This species is classified as *Ictalurus* based on shared pectoral spine anatomy with other *Ictalurus* species (Lundberg, 1975), although Divay & Murray (2015) could not confirm the presence of *Ictalurus* in Eocene deposits of the Cypress Hills Formation. Fossil *Ictalurus* also have been identified from the Brule Formation of South Dakota, which is similar in age, 30–32 mya (J.G. Lundberg pers. comm. 2022). Fossil records of all extant *Ameiurus* species (except *Ameiurus platycephalus*), *Ictalurus dugesii*, *Ictalurus furcatus*, *Ictalurus punctatus*, and *Pylodictis olivaris* were used to calibrate each respective species. These fossils were listed in Lundberg (1975, 1992) and used by Arce H. et al. (2016) to construct their phylogeny.

According to Murray & Holmes (2021), †*Eomacrones wilsoni*, from Late Paleocene deposits (56.0–59.2 mya) in Africa, represents the oldest species belonging to Bagridae based on cranial ornamentation, which we used to calibrate our bagrid outgroups: *Bagrus ubangensis* and *Hemibagrus wyckioides*. Older fossils (59.2–66 mya) from China have been assigned to Bagridae (e.g., Wang et al., 1981), but have not been critically evaluated. To calibrate our outgroup representatives of Clariidae, *Clarias batrachus* and *Channallabes apus*, we used the oldest record of Clariidae fossils found in Africa in the Lower Eocene (Gayet & Meunier, 2003; Jansen et al., 2006; Kappas et al., 2016). The Eocene fossil species †*Chrysichthys mahengeensis* was used to calibrate *Chrysichthys cranchii*. This is the oldest fossil species known of Claroteidae, and was assigned to *Chrysichthys* based on shared dorsal- and pectoral-spine morphology with extant congeners (Murray & Budney, 2003; Sullivan et al., 2008; Murray and Holmes, 2021). We calibrated our outgroup representatives of Mochokidae, *Chiloglanis occidentalis* and *Microsynodontis batesii*, using fossilized *Synodontis* remains dating from the Miocene (Priem, 1920; Pinton et al., 2011). The morphology of the supraoccipital collected from Egypt closely resembles those of living *Synodontis* species (Priem, 1920; Pinton et al., 2011). Lastly, we used †*Pangasius indicus* from the Eocene Sangkarewang Formation, 33.7–54.8 mya (*sensu* Fatimah and Ward, 2009; Zonneveld et al., 2012; Murray, 2019) to calibrate our four representatives of Pangasiidae: *Helicophagus waandersii*, *Pangasianodon hypophthalmus*, *Pangasius larnaudii*, and *Pseudolais pleurotaenia*. Specific fossil information, including age and references, are in Table 1.

Table 1
List of first-occurrence fossils used to time-calibrate Ictaluridae clades and siluriform outgroups for a divergence-time analysis using BEAST2 2.6.3.

Fossil Species	Age (mya) [1]	Clade/Species Calibrated	Reference(s)
Ameiurus brunneus	1.0–1.5	*Ameiurus brunneus*	Lundberg, 1975, 1992
Ameiurus catus	0.0–0.11	*Ameiurus catus*	Lundberg, 1975, 1992
Ameiurus melas	0.5–2.5	*Ameiurus melas*	Lundberg, 1975, 1992
Ameiurus natalis	0.0–1.5	*Ameiurus natalis*	Lundberg, 1975, 1992
Ameiurus nebulosus	0.0–1.5	*Ameiurus nebulosus*	Lundberg, 1975, 1992
†*Ameiurus pectinatus*	34.0–34.2	*Ameiurus*	Lundberg, 1975, 1992; Evanoff et al., 2001
Ameiurus serracanthus	1.0–1.5	*Ameiurus serracanthus*	Lundberg, 1975, 1992
Ictalurus dugesii	1.0–1.5	*Ictalurus dugesii*	Lundberg, 1975, 1992
Ictalurus furcatus	1.0–1.5	*Ictalurus furcatus*	Lundberg, 1975, 1992
Ictalurus punctatus	15.9–18.9	*Ictalurus punctatus*	Tedford et al., 1987; Lundberg, 1975, 1992
†*Ictalurus rhaeas*	30.0–37.0	*Ictalurus*	Cope, 1891; Lundberg, 1975
Pylodictis olivaris	15.9–18.9	*Pylodictis olivaris*	Tedford et al., 1987; Lundberg, 1975, 1992
Clariidae sp.	34.0–56.0	Clariidae	Gayet & Meunier, 2003
†*Chrysichthys mahengeensis*	45.66–46.0	Claroteidae	Harrison et al., 2001; Murray & Budney, 2003
†*Eomacrones wilsoni*	56.0–59.2	Bagridae	Murray & Holmes, 2021
†*Pangasius indicus*	33.7–54.8	Pangasiidae	Fatimah & Ward, 2009; Zonneveld et al., 2012; Murray, 2019
Synodontis sp.	~18	Mochokidae	Priem, 1920; Pinton et al., 2011

[1] mya = million years ago.

To construct our time-calibrated phylogeny, we performed two MCMC analyses for 250 million generations. In both analyses, trees were sampled every 10,000 generations. We assessed the effective sample size (ESS) and convergence for both analyses using Tracer 1.7.1 (Rambaut et al., 2018). We discarded the first 10% of trees as burn-in and summarized the remaining trees using TreeAnnotator 2.6.3 (Bouckaert et al., 2019). Lastly, we visualized our time-calibrated phylogeny using FigTree 1.4.4.

3. Results

We constructed a ML phylogeny (Fig. 2) using a concatenated DNA sequence alignment of 24,470 nucleotide base pairs. The family Ictaluridae was strongly supported as monophyletic with a 100 BS value. Within Ictaluridae, non-monotypic genera *Ameiurus*, *Ictalurus* and *Noturus* were also strongly supported as monophyletic (100 BS for *Ameiurus* and *Noturus*, 99 BS for *Ictalurus*). *Noturus* was sister to all other genera followed by *Pylodictis* as sister to a clade composed of *Ameiurus*, *Ictalurus*, *Prietella*, and *Trogloglanis* (98 BS). Within that clade (94 BS), we found two strongly supported subclades: (1) *Ameiurus* (*P. phreatophila* + *Trogloglanis pattersoni*) (100 BS) and (2) *Ictalurus* + *P. lundbergi* (95 BS). Within the first subclade, *P. phreatophila* was strongly supported as sister to *T. pattersoni* (100 BS); these hypogean species were sister to the epigean genus *Ameiurus* (100 BS). In the second clade, the hypogean *P. lundbergi* was sister to the epigean genus *Ictalurus* (95 BS). Thus, *Prietella* was polyphyletic with *P. phreatophila* and *P. lundbergi* descending from different common ancestors shared with the surface-dwelling relatives *Ameiurus* and *Ictalurus*, respectively. Furthermore, the polyphyletic status of *Prietella* indicated that troglobitic ictalurids do not form a monophyletic clade.

Both independent MCMC runs of our fossil-calibrated phylogeny (Fig. 3) achieved convergence with strong ESS likelihood scores > 200. The topology of our fossil-calibrated phylogeny was consistent with our ML phylogeny, placing *Noturus* as sister to all other genera (1.0 posterior probability (PP)), followed by *Pylodictis* as sister to a clade composed of *Ameiurus*, *Ictalurus*, *Prietella*, and *Trogloglanis* (1.0 PP). The placement of the three cave species was also consistent with our ML phylogeny: (1) *P. phreatophila* formed a sister pair with *T. pattersoni* (1.0 PP), which was sister to *Ameiurus* (1.0 PP), and (2) *P. lundbergi* was sister to *Ictalurus* (0.99 PP).

With respect to our fossil-calibration analysis, we estimated the origin of crown group Ictaluridae to ~ 60 mya, with a 95% confidence interval (CI) of 47–74 mya. Thus, ictalurids originated sometime between the Late Cretaceous and the Eocene. Within Ictaluridae, the ancestor of *Noturus* began to diversify ~ 32 mya (24–39 mya 95% CI). Between the Paleocene and the Eocene, *Pylodictis* diverged from the ancestor of *Ameiurus* + *Ictalurus* + *Prietella* + *Trogloglanis* ~ 54 mya (43–65 mya 95% CI). The ancestor of these remaining genera began to diversify shortly afterwards, ~52 mya (42–62 mya 95% CI). During the Eocene ~ 40 mya (34–48 mya 95% CI), the common ancestor of *P. phreatophila* and *T. pattersoni* diverged from the ancestor of *Ameiurus*; *P. phreatophila* split from *T. pattersoni* ~ 28 mya (19–37 mya 95% CI). The ancestor of *Ameiurus* began to diversify ~ 23 mya (17–30 mya 95% CI). *Prietella lundbergi* split from the ancestor of *Ictalurus* ~ 43 mya (33–54 mya 95% CI), between the Eocene and Oligocene. Lastly, the ancestor of *Ictalurus* began to diversify ~ 28 mya (23–36 mya 95% CI).

4. Discussion

4.1. Evolutionary relationships of cave ictalurids

We constructed both a ML phylogeny and a fossil-calibrated phylogeny of Ictaluridae using the largest molecular dataset of any study to date, as well as the first inclusion of molecular data from *Trogloglanis pattersoni*. The topologies of our two phylogenies were consistent, and the positions of each cave species and of the surface-dwelling genera were strongly supported. We found that the hypogean taxa *Prietella lundbergi*, *P. phreatophila*, and *T. pattersoni* did not form a monophyletic clade. Moreover, *P. phreatophila* and *T. pattersoni* were recovered as sister taxa. Thus, these two species may have diverged as a result of a subterranean dispersal event, which we discuss below. The polyphyletic nature of the Troglobites clade proposed by Arce H. et al. (2016) supports our hypothesis that the troglobitic ictalurids have evolved in parallel, resulting from a minimum of two cave colonization events by surface-dwelling ancestors. Furthermore, the placement of *P. lundbergi* as sister to *Ictalurus*, and *P. phreatophila* + *T. pattersoni* as sister to *Ameiurus*, indicates that *Prietella* is a polyphyletic genus. This supports our prediction: *Prietella* should be restricted to *P. phreatophila* (type species) and *P. lundbergi* requires generic reclassification.

The phylogenetic placement of the three cave species included in our analyses was congruent with previous molecular phylogenies of Ictaluridae. Egge & Simons (2009) observed a sister relationship between *Ameiurus melas* and *P. phreatophila* in their MP and BI molecular-only and BI molecular + morphological phylogenies of *Noturus*. Our results are related to those of Egge & Simons (2009) because we used the same *cyt b* and *rag2* sequences downloaded from GenBank. Our addition of *co1* did not affect the previously observed relationship between *Ameiurus* and *Prietella phreatophila*. Egge & Simons (2009) alternatively found a sister-group relationship between *P. phreatophila* and *Noturus* in their MP phylogeny combining morphological and molecular data. Wilcox et al. (2004) observed sister relationships between *P. lundbergi* + *Ictalurus* and *P. phreatophila* + *Ameiurus* in their BI and ML analyses, consistent with our results, which again was expected because we used the same

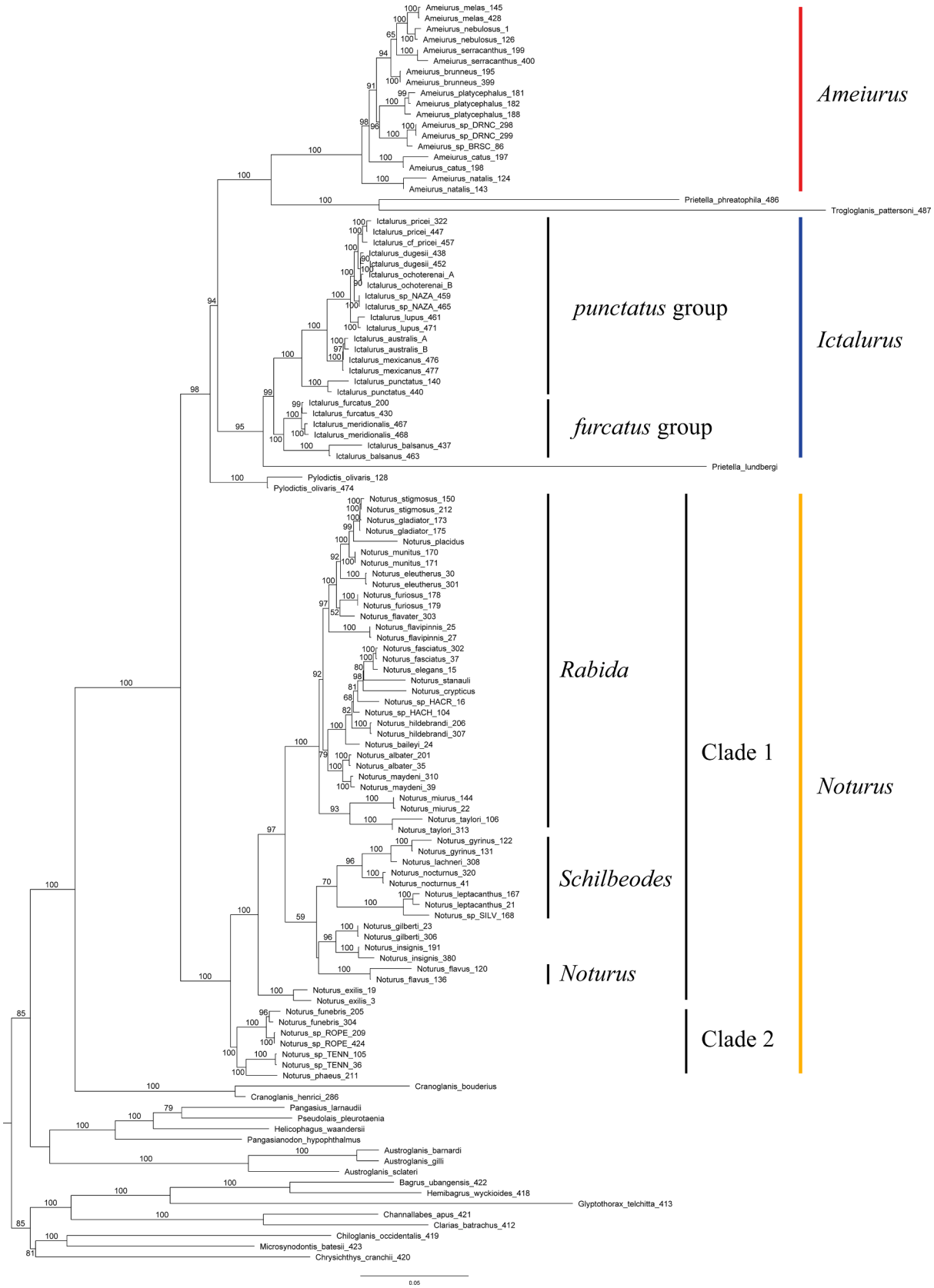

Fig. 2. Maximum likelihood phylogeny of Ictaluridae based on an 11-region concatenated data matrix analyzed with IQ-TREE. Bootstrap values ≥ 50% are presented above each branch.

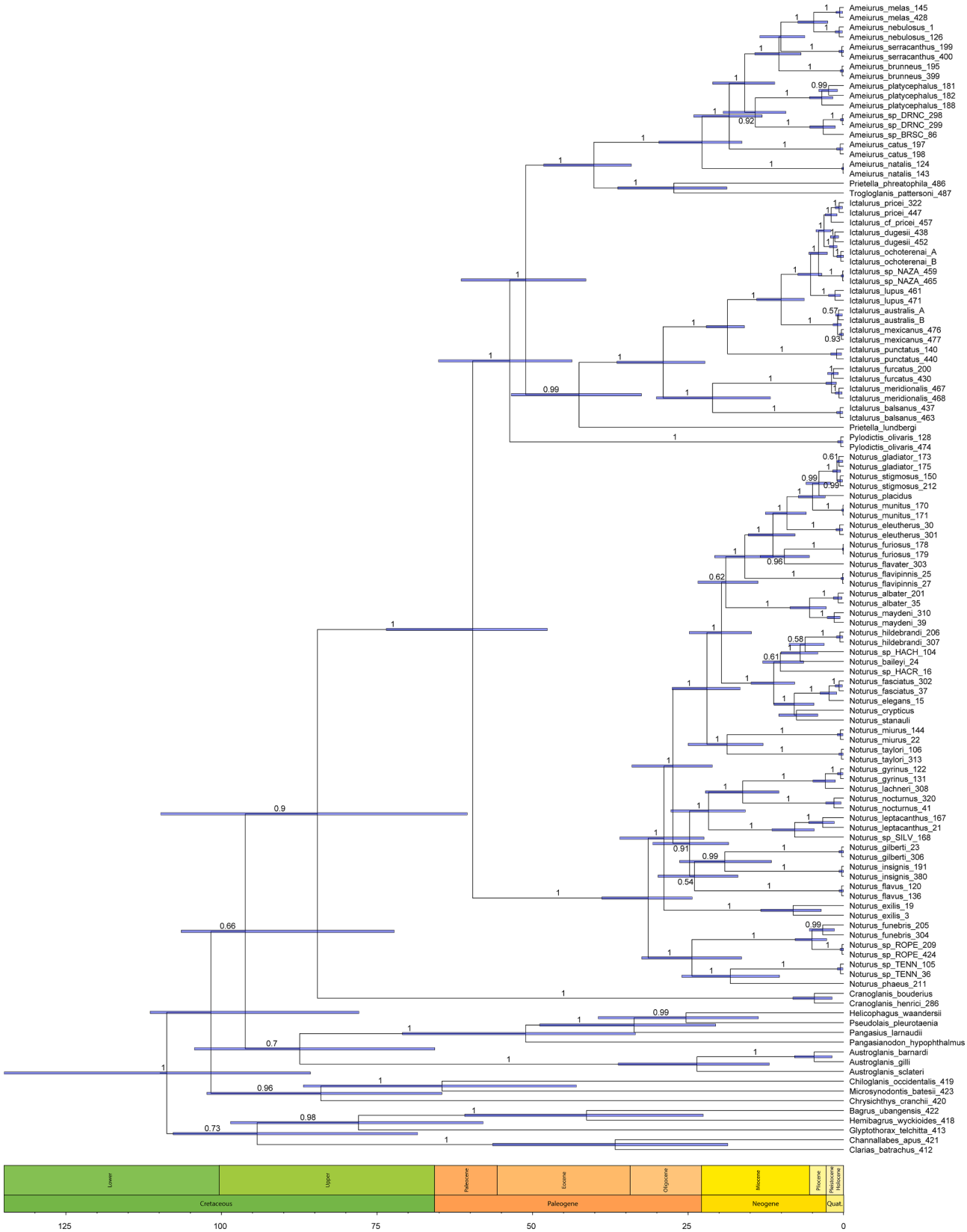

Fig. 3. Fossil-calibrated phylogeny of Ictaluridae using the CladeAge package in BEAST2. Divergence-time estimates were calculated using 16 fossil specimens. Posterior probabilities ≥ 0.5 are presented above each branch. Blue node bars represent the 95% confidence interval of estimated origin times. Geological scale axis measured in million years before present. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mitochondrial genes for *P. lundbergi* and *P. phreatophila* downloaded from GenBank. We included additional genes for *P. phreatophila* in our analysis, and these genes supported the same relationship. Arce H. et al. (2016), however, found the four troglobitic species to be monophyletic in their MP phylogeny combining molecular and morphological data. These results may be due to a combination of using convergent morphological characters as well as long-branch attraction of the highly divergent troglobites, as suggested by Wilcox et al. (2004). Long-branch attraction occurs when two distantly-related lineages are erroneously grouped together based on convergent similarities, such as identical amino acids acquired independently due to finite combinations of nucleotides (Bergsten, 2005; Susko & Roger, 2021). The more differences that accrue in long-branch lineages, the higher the likelihood of sharing similarities with distantly related lineages (Bergsten, 2005). MP phylogenetic analyses are the most susceptible to long-branch attraction (Wilcox et al., 2004; Bergsten, 2005) and they were the only phylogenetic analyses to recover the troglobitic species as monophyletic (Wilcox et al., 2004; Arce H. et al., 2016).

Some early morphological studies also support the placement of the cave species included in our study. Hubbs & Bailey (1947) suggested that *T. pattersoni* most likely diverged from *Ameiurus*, which was its closest relative at the time given that *P. phreatophila* had not yet been described. Also, Suttkus (1961) proposed *P. phreatophila* to be closely related to *Ictalurus*, which at the time also included *Ameiurus* as a subgenus. Furthermore, caudal fin morphology supports our placements of both *Prietella* species. The emarginate caudal fin observed in *P. lundbergi* more closely resembles *Ictalurus*, whereas the more truncated and rounded caudal fin observed in *P. phreatophila* more closely resembles *Ameiurus* (Walsh & Gilbert, 1995; Wilcox et al., 2004).

4.2. Topology of Ictaluridae

Of the many phylogenies of Ictaluridae, we are the first to recover *Noturus* as the sister clade to all other ictalurids. Previous studies have placed *Ameiurus* (Hardman & Hardman, 2008), *Ictalurus* (Taylor, 1969; Lundberg, 1992; Egge & Simons, 2009), and the Troglobites clade (Arce H. et al., 2016) as sister to all remaining genera. Prior to Arce H. et al. (2016), taxonomic coverage in phylogenetic studies was incomplete; the cave species as well as most Mexican *Ictalurus* species were often missing. With respect to Arce H. et al. (2016), the inclusion of potentially convergent morphological characters available for *Prietella*, *Satan*, and *Trogloglanis*, as well as long-branch attraction may have contributed to the unique placement of the troglobitic ictalurid genera within the family. We believe our novel findings more accurately reflect relationships among extant Ictaluridae given the near-complete taxon sampling and the use of the largest molecular-only dataset of any study to date. This applies to the discrepancies between our phylogeny and previous work for each of the genera discussed below.

4.2.1. Topology of Noturus

Within *Noturus*, the most species-rich ictalurid genus, Taylor (1969) grouped species into three subgenera (Table 2) based on morphological similarities: monotypic *Noturus* (*N. flavus*), *Rabida* (containing the *elegans*, *furiosus*, *hildebrandi*, and *miurus* species groups), and *Schilbeodes* (containing the *funebris* species group). *Noturus* phylogenies by Hardman (2004), Near & Hardman (2006), Hardman & Hardman (2008), and Egge & Simons (2009) supported *Rabida* as monophyletic, but not *Schilbeodes*. Egge & Simons (2009) considered Taylor's subgenera to be untenable and proposed seven phylogenetic clades unassigned to Linnean rank: *albater*, *elegans*, *funebris*, *furiosus*, *gyrinus*, *hildebrandi*, and *rabida,* the last one comprising the *albater*, *elegans*, *furiosus*, and *hildebrandi* subclades (Table 2).

Our phylogenetic analyses similarly supported the monophyly of Taylor's (1969) subgenus *Rabida*, but not his *Schilbeodes*. The subgenus *Schilbeodes* is therefore restricted here to four species: *N. gyrinus* (type species), *N. lachneri*, *N. leptacanthus* and *N. nocturnus*. With respect to the groups and subclades proposed by Taylor (1969) and Egge & Simons (2009), respectively, our results were largely consistent. For example, our analysis supported Egge & Simons' *furiosus* subclade which consolidated the *furious* and *miurus* groups of Taylor, as well as their *elegans*

Table 2
Comparison of *Noturus* subgenus and species group classifications proposed by Taylor (1969), Egge & Simons (2009), and the current study.

		Current Study	Taylor 1969	Egge & Simons 2009	
Nominal Species (* denotes type species of respective subgenus)	Molecular Clade	Subgenus (Group)	Subgenera	Group	Clades (Subclades)
Noturus albater	Clade 1.1.1	*Rabida* (*albater*)	*Rabida*	—	*rabida* (*albater*)
Noturus maydeni	Clade 1.1.1	*Rabida* (*albater*)	*Rabida* [as *N. albater*]	—	*rabida* (*albater*)
Noturus eleutherus	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	—	*rabida* (*furiosus*)
Noturus flavater	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	*miurus*	*rabida* (*furiosus*)
*Noturus furiosus**	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	*furiosus*	*rabida* (*furiosus*)
Noturus gladiator	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida* [as *N. stigmosus*]	*furiosus* [as *N. stigmosus*]	*rabida* (*furiosus*)
Noturus munitus	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	*furiosus*	*rabida* (*furiosus*)
Noturus placidus	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	*furiosus*	*rabida* (*furiosus*)
Noturus stigmosus	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	*furiosus*	*rabida* (*furiosus*)
Noturus flavipinnis	Clade 1.1.1	*Rabida* (*furiosus*)	*Rabida*	*miurus*	*rabida* (*furiosus*)
Noturus miurus	Clade 1.1.1	*Rabida* (*miurus*)	*Rabida*	*miurus*	*rabida* (*furiosus*)
Noturus taylori	Clade 1.1.1	*Rabida* (*miurus*)	—	—	*rabida*
Noturus baileyi	Clade 1.1.1	*Rabida* (*elegans*)	*Rabida*	*hildebrandi*	*rabida* (*elegans*)
Noturus crypticus	Clade 1.1.1	*Rabida* (*elegans*)	*Rabida* [as *N. elegans*]	*elegans* [as *N. elegans*]	*rabida* (*elegans*)
Noturus elegans	Clade 1.1.1	*Rabida* (*elegans*)	*Rabida*	*elegans*	*rabida* (*elegans*)
Noturus fasciatus	Clade 1.1.1	*Rabida* (*elegans*)	*Rabida* [as *N. elegans*]	*elegans* [as *N. elegans*]	*rabida* (*elegans*)
Noturus hildebrandi	Clade 1.1.1	*Rabida* (*elegans*)	*Rabida*	*hildebrandi*	*rabida* (*elegans*)
Noturus stanauli	Clade 1.1.1	*Rabida* (*elegans*)	—	—	*rabida* (*elegans*)
Noturus trautmani	—	*Rabida* (*elegans*)	*Rabida*	*elegans*	*rabida* (*elegans*)
*Noturus gyrinus**	Clade 1.1.2	*Schilbeodes*	*Schilbeodes*	—	*gyrinus*
Noturus lachneri	Clade 1.1.2	*Schilbeodes*	*Schilbeodes*	—	*gyrinus*
Noturus leptacanthus	Clade 1.1.2	*Schilbeodes*	*Schilbeodes*	—	*funebris*
Noturus nocturnus	Clade 1.1.2	*Schilbeodes*	*Schilbeodes*	—	—
*Noturus flavus**	Clade 1.1.2	*Noturus*	*Noturus*	—	—
Noturus gilberti	Clade 1.1.2	—	*Schilbeodes*	—	—
Noturus insignis	Clade 1.1.2	—	*Schilbeodes*	—	—
Noturus exilis	Clade 1.2	—	*Schilbeodes*	—	—
Noturus funebris	Clade 2	— (*funebris*)	*Schilbeodes*	*funebris*	*funebris*
Noturus phaeus	Clade 2	— (*funebris*)	*Schilbeodes*	*funebris*	—

subclade which combined Taylor's *elegans* and *hildebrandi* groups.

Discrepancies between our tree and previous ones include the placements of *N. leptacanthus* and *N. phaeus*. Our analyses did not support a sister group relationship between *N. leptacanthus* and *N. funebris*, the only two members of the *funebris* clade proposed by Egge & Simons (2009) and likewise supported by Hardman & Hardman (2008). Instead, our analyses placed *N. leptacanthus* sister to a clade composed of *N. gyrinus*, *N. lachneri*, and *N. nocturnus* with all four species comprising the subgenus *Schilbeodes* as restricted herein. Regarding *N. phaeus*, our analyses placed this species in a clade with *N. funebris* and two potentially undescribed species. Taylor (1969) likewise proposed a close relationship between *N. funebris* and *N. phaeus*, the only two members of his *funebris* group of *Schilbeodes*. Our results support his group, but not its placement in subgenus *Schilbeodes* as restricted here. The placement of *N. phaeus* varied among phylogenetic analyses by Egge & Simons (2009). Their MP and BI analyses of morphological data supported a sister group relationship between *N. phaeus* and *N. funebris* (Egge & Simons, 2009: F**ig. 6), consistent with Taylor (1969) and our study. Their MP analyses of molecular data and combined molecular and morphological data placed *N. phaeus* sister to *N. funebris* + *N. leptacanthus* (Egge & Simons, 2009: F**igs. 7A, 8A). Finally, their BI analyses of molecular data and combined data upheld *N. funebris* + *N. leptacanthus*, but did not support its close relationship with *N. phaeus* (Egge & Simons, 2009: F**igs. 7B, 8B).

Among the 29 valid species of *Noturus*, only five remain here unassignable to nominal subgenera: *N. exilis*, *N. funebris*, *N. gilberti*, *N. insignis,* and *N. phaeus*. Taylor (1969) placed all these species in *Schilbeodes*; however, our results restrict this subgenus to *N. gyrinus* (type species), *N. lachneri*, *N. leptacanthus*, and *N. nocturnus.* As mentioned above, there is strong morphological and molecular support for a sister group relationship between *N. funebris* and *N. phaeus* (Taylor, 1969; Egge & Simons, 2009). Our molecular analysis places *N. funebris*, *N. phaeus*, and two potentially undescribed species in a clade (Clade 2) sister to one containing all other species of *Noturus* (Clade 1). According to our results, the first species to diverge in Clade 1 is *N. exilis*, a wide-ranging monophyletic species with geographically isolated populations in the Eastern and Interior Highlands and the previously glaciated Central Lowlands (Blanton et al., 2013).

The remaining members of Clade 1 are divided among two subclades, one corresponding to the subgenus *Rabida* (Clade 1.1.1) and the other (Clade 1.1.2) comprising the subgenus *Schilbeodes* (four species), *N. flavus* (monotypic subgenus *Noturus*), *N. gilberti*, and *N. insignis* (unassigned here). Although our analyses strongly support the monophyly of subgenus *Schilbeodes sensu stricto* in our fossil-calibrated phylogeny (1.0 PP), relationships involving *N. flavus* and *N. gilberti* + *N. insignis* remain poorly resolved. Similar to our results, previous studies provided weak support for a clade composed of *N. flavus*, *N. gilberti* and *N. insignis* (Hardman & Hardman, 2008; Egge & Simons, 2009). Our study independently corroborates these results because we used novel genetic sequence data for these species. The potential for a close relationship between *N. flavus* and *N. insignis* is biogeographically intriguing. The two species are mostly allopatric, with *N. flavus* widely distributed throughout the Mississippi and Great Lakes-St. Lawrence basins and *N. insignis* common to Atlantic slope drainages from New York to Georgia (Taylor, 1969). *Noturus gilberti*, on the other hand, is restricted to the upper Roanoke drainage in Virginia and North Carolina where it may co-occur with *N. insignis* (Jenkins & Burkhead, 1994), its sister species.

4.2.2. Topology of Ictalurus

In early molecular phylogenies, *Ictalurus* was limited to species distributed in the USA and Canada, including *I. furcatus*, *I. lupus*, and *I. punctatus* (Hardman, 2004; Wilcox et al., 2004; Hardman & Hardman, 2008). Arce H. et al. (2016) were the first to consider Mexican species in a phylogenic analysis that included genetic sequences for *I. balsanus*, *I. meridionalis*, and *I. pricei*. Their results provide evidence for a deep divergence within extant *Ictalurus* dividing *I. furcatus* + *I. meridionalis* from all other *Ictalurus* except *I. balsanus*. Rodiles-Hernández et al. (2010) previously supported the same relationship based on morphology. Our results support the same split except with *I. balsanus* sister to *I. furcatus* + *I. meridionalis* based on new genetic sequence data absent from Arce H. et al. (2016). Among the remaining *Ictalurus*, *I. punctatus* was sister to a clade composed of *I. australis* + *I. mexicanus*, *I. lupus*, *I. dugesii* + *I. ochoterenai*, *I. pricei*, and a potentially new species from the Nazas River, Mexico.

Pérez-Rodríguez et al. (2022) constructed a molecular phylogeny of *Ictalurus*, the first to include specimens of all nominal species within the genus. Similar to our study, they found a deep divergence between the *I. balsanus*, *I. furcatus*, and *I. meridionalis* clade (the *furcatus* group) and all remaining extant *Ictalurus* species (the *punctatus* group). Pérez-Rodríguez et al. (2022) found very low genetic divergence between three pairs of species: *I. australis* + *I. mexicanus*, *I. dugesii* + *I. ochoterenai* and *I. furcatus* + *I. meridionalis*. Lundberg (1992) considered *I. mexicanus* distinct, but noted that *Ictalurus australis*, *I. ochoterenai*, and *I. meridionalis* may be conspecific with *I. punctatus*, *I. dugesii*, and *I. furcatus*, respectively. Rodiles-Hernández et al. (2010) alternatively supported the taxonomic distinctiveness of *I. furcatus* and *I. meridionalis* based on 12S/16S mitochondrial genes and morphological traits, such as pectoral spine ornamentation, anal-fin ray and vertebrae counts, and differences in the supraoccipital process. Our study corroborates the results of Pérez-Rodríguez et al. (2022), showing low genetic divergence between the species within each pair; however, we used the same sequence data for *I. australis* and *I. ochoterenai* downloaded from GenBank.

Pérez-Rodríguez et al. (2022) also found both *I. pricei* and *I. lupus* formed species complexes; each complex comprised distinct lineages that may represent potentially undescribed species. Three of these lineages were included within our phylogeny: *Ictalurus* sp. NAZA from the Nazas River, *Ictalurus* cf. *pricei* from the Mezquital River, and *I. lupus* from the Conchos River. Further study, including morphological analyses, are necessary to determine whether these lineages require formal species descriptions. Finally, Pérez-Rodríguez et al. (2022) estimated *Ictalurus* began to diversify in the Oligocene, which overlaps with our somewhat older estimate between the Oligocene and Eocene.

4.2.3. Topology of Ameiurus

With respect to *Ameiurus*, our ML and time-calibrated phylogenies both placed *Ameiurus natalis* as sister to all other extant species within the genus based on novel genetic sequence data for all *Ameiurus* species. This result is consistent with the findings of Hardman & Page (2003), Hardman & Hardman (2008), and Arce H. et al. (2016). Our ML and time-calibrated phylogenies supported *A. catus* as sister to the remaining species of *Ameiurus*. This is consistent with Arce H. et al. (2016), but differs from Hardman & Page (2003) and Hardman & Hardman (2008) wherein *A. catus* is sister to *A. platycephalus.* Our study and previous ones based on morphology (Lundberg, 1992), molecules (Hardman & Hardman, 2008) or both (Arce H. et al., 2016) all support a close relationship between *A. melas* and *A. nebulosus*, however only the molecular phylogenies place these species in a crown clade with *A. brunneus* and *A. serracanthus*. Within this crown clade, *A. brunneus* was the first species to diverge in our analyses vs. *A. serracanthus* in Hardman & Hardman (2008). That said, our placement of *A. serracanthus* as sister to *A. melas* + *A. nebulosus* was poorly supported (65 BS, <0.5 PP). Alternatively, Arce H. et al. (2016) supported a clade composed of *A. serracanthus*, *A. brunneus*, and the extinct fossil species †*A. peregrinus.*

Surprisingly, we identified a potentially new species of *Ameiurus* from two Atlantic slope drainages, the Cape Fear and Santee of North and South Carolina, respectively. This species-level lineage was sister to a clade of three individuals of *A. platycephalus* from the Haw River (Cape Fear Basin), a Broad River tributary (Santee Basin), and Stevens Creek (Savannah Basin), respectively. *Ameiurus platycephalus* is native to Atlantic slope drainages and its range broadly overlaps with that of

A. brunneus, a similar looking species (Yerger & Relyea, 1968; Tracy et al., 2020). In our analysis, *A. brunneus* was represented by two individuals from separate tributaries to the Chattahoochee River (Apalachicola Basin), outside of the native range of *A. platycephalus*. Our results suggest that it is time to revisit relationships among the Atlantic and Gulf slope *Ameiurus* formerly known as "flat-headed" bullheads (Yerger & Relyea, 1968), namely *A. brunneus*, *A. platycephalus* and *A. serracanthus*.

4.3. Divergence-time estimations of Ictaluridae

We estimated that Ictaluridae originated sometime between the Late Cretaceous and Eocene, ~47–74 mya. This proposed origin time is consistent with the findings of Hardman & Hardman (2008), who proposed that Ictaluridae began to diversify ~ 59–72 mya in the Late Cretaceous to Paleocene. Kappas et al. (2016) similarly suggested that Ictaluridae originated ~ 63–72.5 mya. Larger phylogenies of Siluriformes have estimated younger origin times, albeit with the inclusion of only 1–3 representatives of Ictaluridae. For example, Lundberg et al. (2007) estimated that Ictaluridae originated ~ 37.9–42.7 mya. In their phylogeny of bony fishes (Osteichthyes), Betancur-R et al. (2017) estimated that Ictaluridae began to diverge ~ 30–35 mya. Arce H. et al. (2016) also proposed a relatively young origin for Ictaluridae during the Eocene, ~33.9–56 mya. We used the same ictalurid fossils as Arce H. et al. (2016) to calibrate our phylogeny with the notable exception of the extinct genus †*Astephus*, the oldest fossil to be considered an ictalurid (Buchheim & Surdam, 1977; Grande & Lundberg, 1988; Lundberg, 1992). In the phylogeny of Arce H. et al. (2016), †*Astephus* was nested within the outgroup lineages (vs. the sister lineage of extant ictalurids), prompting them to elevate this group from a subfamily of Ictaluridae to the rank family †Astephidae. Therefore, we did not include †*Astephus* in our analyses, which may partially account for our older estimation of Ictaluridae.

With respect to the troglobitic ictalurids, Arce H. et al. (2016) proposed that this monophyletic clade diverged from the surface-dwelling confamilials ~ 47 mya during the Eocene and began to diversify ~ 9 mya during the Miocene. Our time estimates for the divergence of cave species from surface-dwelling relatives overlapped with those of Arce H. et al. (2016); however, our results support two independent origins of cave-dwelling species within Ictaluridae. We propose that *P. phreatophila* + *T. pattersoni* diverged from *Ameiurus* during the Eocene (~34–48 mya) and that *P. lundbergi* diverged from *Ictalurus* during the Eocene to Oligocene (~33–54 mya).

4.4. Evolutionary history of troglobitic ictalurids

The hypothesis that cave-dwelling catfish species evolved in parallel within Ictaluridae is intuitive given the allopatric contemporary distribution of their insular habitats (Wilcox et al., 2004; Burr et al., 2020). The significant geological barriers separating these species, especially between the two *Prietella* species, suggests that subterranean radiation of a common troglobitic ancestor is unlikely (Hendrickson et al., 2001; Wilcox et al., 2004). That said, our evidence supported a sister relationship between *P. phreatophila* and *T. pattersoni*, which implies that subterranean dispersal events and subsequent speciation are possible. It is unsurprising that repeated cave-colonization events likely occurred within Ictaluridae. Many epigean ictalurids are preadapted to subterranean life (e.g., poor eyesight, nocturnal habits) and typically rely on other senses, such as touch, taste, and electroreception, to navigate and acquire food (Eigenmann, 1919; Langecker & Longley, 1993; Burr et al., 2020). Furthermore, some surface-dwelling species (*Ameiurus nebulosus*, *A. natalis*, and *I. punctatus*) partially live within caves and concentrate around cave entrances (Hale & Streever, 1994; Poly, 2001). The existence of these troglophilic populations and the sister relationships of cave species with surface-dwelling relatives further supports our hypothesis that ictalurids colonized subterranean habitats at least twice during their evolutionary history.

Our results indicate that *Prietella lundbergi* diverged from the common ancestor of *Ictalurus* sometime during the Paleocene-Eocene. The long distance and extensive geological barriers between *P. lundbergi* and *P. phreatophila* suggest that a subterranean divergence between these two species is unlikely (Hendrickson et al., 2001; Wilcox et al., 2004), whereas divergence from a surface-dwelling ancestor potentially resembling extant *Ictalurus* is more probable. This is further supported by the occurrence of epigean species of *Ictalurus* near *P. lundbergi* (Wilcox et al., 2004; Burr et al., 2020) and documented instances of *Ictalurus punctatus* living within caves (Hale & Streever, 1994). In contrast, the native distribution of *Ameiurus* (the closest relative of *P. phreatophila* + *T. pattersoni*) does not overlap with *P. lundbergi* (Wilcox et al., 2004; Burr et al., 2020). It is possible that during the Paleocene and/or Eocene, surface waters were connected with the subterranean springs in the Tamesí River drainage where *P. lundbergi* currently occurs (Walsh & Gilbert, 1995; Hendrickson et al., 2001). This may have allowed for the dispersal and subsequent selection of individuals better adapted for subterranean life. Magmatic activity as well as orogenesis in Mexico in the Late Cenozoic have been linked to the fragmentation of freshwater habitats and vicariant speciation (González-Rodríguez et al., 2013; Fitz-Díaz et al., 2018). Such activity may have disrupted connectivity between the subterranean springs and surface waters, further reinforcing the ancient reproductive isolation of the subterranean population. Alternatively, it is possible that parapatric speciation occurred as a result of *in situ* ecological specialization followed by reproductive isolation of the ancestral *P. lundbergi* population rather than vicariance from surface populations (Plath & Tobler, 2010). Whichever the case, *P. lundbergi* appears to have been isolated from the ancestral stock of *Ictalurus* for at least 34 million years.

Our phylogeny suggests that the common ancestor of *Prietella phreatophila* + *Trogloglanis pattersoni* diverged from ancestral *Ameiurus* during the Eocene. A surface-dwelling ancestor similar to *Ameiurus* may have colonized subterranean waters somewhere near either the current distribution of *P. phreatophila* in northern Coahuila (Walsh & Gilbert, 1995; Hendrickson et al., 2001) or the Edwards Aquifer in Texas containing *T. pattersoni* (Langecker & Longley, 1993; Walsh & Gilbert, 1995). Similarly with *P. lundbergi* and *Ictalurus*, *Ameiurus* species currently occur in surface waters near both *P. phreatophila* and *T. pattersoni* (Wilcox et al., 2004; Burr et al., 2020), whereas contemporary *P. lundbergi* is geographically distant from the other cave species. In addition, populations of *A. natalis* and *A. nebulosus* have been documented living within caves (Hale & Streever, 1994; Poly, 2001). These occurrences, as well as our phylogenetic results, support the hypothesis that an *Ameiurus*-like ancestor invaded subterranean waters and subsequently diverged into *P. phreatophila* and *T. pattersoni*.

Prietella phreatophila split from *T. pattersoni* sometime between the Eocene and Miocene. Although the extent of connectivity is currently unknown between locations where *P. phreatophila* and *T. pattersoni* occur (Sanchez et al., 2016; Sanchez et al., 2018b), their shared common ancestor suggests that speciation may have resulted from a subterranean dispersal event between aquifers. The ancestral species may have travelled through the Knippa Gap, a hydrological restriction point east of the San Antonio Pool in Texas where *T. pattersoni* and *S. eurystomus* occur (Adkins, 2013; Green et al., 2019). Hydrological connectivity through the Knippa Gap is variable, depending on groundwater levels (Green et al., 2019). This transient barrier and others may have been sufficient to restrict frequent movement between the ancestral Mexican and American populations, facilitating speciation. Additional evidence that supports the connectivity of these aquifers is the occurrence of *P. phreatophila* populations in northern Coahuila and the Amistad National Recreation Area, Texas (Walsh & Gilbert, 1995; Hendrickson et al., 2001; Hendrickson et al., 2017; Krejca & Reddell, 2019). The aquifer in northern Coahuila is separated from the Amistad Recreational Area by the Rio Grande, yet *P. phreatophila* is found both north and south of the river (Krejca & Reddell, 2019). The distribution of *P. phreatophila*

and our results imply that the Edwards-Trinity Plateau and the aquifer in northern Coahuila were likely hydrologically connected during the Eocene-Miocene (Krejca & Reddell, 2019). Other troglobitic animals have demonstrated similar distribution patterns of closely related populations. Three isopod species (*Cirolanides texensis*, *Lirceolus cocytus*, and *Mexistenasellus coahuila*), as well as a species of amphipod (*Paraholsingerius smaragdinus*) occur both north and south of the Rio Grande in Mexican and American aquifers (Krejca, 2005; Krejca & Reddell, 2019). These populations are genetically similar to one another, which further supports the existence of hydrological connections between aquifers in Texas and Coahuila (Krejca, 2005; Krejca & Reddell, 2019).

4.5. Satan eurystomus

As previously indicated, we were unable to include *S. eurystomus* in our molecular phylogeny due to tissue samples being unavailable for sequencing. Therefore, we were unable to determine whether *S. eurystomus* is most closely related to *T. pattersoni* with which it co-occurs, or with an extant surface-dwelling relative. *Pylodictis olivaris* may be the closest living relative of *S. eurystomus* given the notable morphological traits both species share (Lundberg et al., 2017). Unlike for *Prietella* and *Trogloglanis*, early morphological studies agreed upon a sister relationship between *S. eurystomus* and *P. olivaris* (Hubbs & Bailey, 1947; Suttkus, 1961; Taylor, 1969; Lundberg, 1970; Lundberg, 1982). Hubbs & Bailey (1947) mentioned that *P. olivaris* may be preadapted for subterranean life, being a light-averse species that commonly hides under rocks and logs. Similar to other ictalurid species, *P. olivaris* relies more on its senses of touch and taste to navigate and obtain food than its eyesight (Hubbs & Bailey, 1947; Burr et al., 2020). If *S. eurystomus* and *P. olivaris* are sister species, this would indicate that ictalurids have independently colonized underground habitats three times, evolving in parallel. Until tissue samples become available for *S. eurystomus*, however, this hypothesis is impossible to test with molecular data.

CRediT authorship contribution statement

Francesco H. Janzen: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Rodolfo Pérez-Rodríguez:** Resources, Data curation, Writing – review & editing, Funding acquisition. **Omar Domínguez-Domínguez:** Resources, Data curation, Writing – review & editing, Funding acquisition. **Dean A. Hendrickson:** Resources, Writing – review & editing, Funding acquisition. **Mark H. Sabaj:** Resources, Data curation, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Gabriel Blouin-Demers:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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