Identity Crisis: An Integrative Re-Evaluation of Typhlatya Shrimp within the Karst Aquifer of the Yucatán Peninsula, Mexico.

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ABSTRACT

The Yucatán Peninsula, Mexico is a carbonate platform well-known for extensive karst networks of densely stratified aquifer ecosystems. This aquifer supports diverse *anchialine* fauna, including species of the globally distributed atyid shrimp genus *Typhlatya*. Four species (*T. campecheae*, *T. pearsei*, *T. dzilamensis* and *T. mitchelli*) are endemic to the Peninsula, of which three are federally listed in Mexico. This first integrative evaluation [i.e., molecular (public and newly generated), morphological, broad geographic and type locality sampling, and environmental data] of Yucatán *Typhlatya* reveals considerable species identity conflict in prior phylogenetic assessments, broad species ranges and sympatry within cave systems, five genetic lineages (three known and two new to science) with the *endangered* *T. campecheae* herein classified as junior synonym of the *vulnerable* *T. pearsei*. Ancestral/divergence reconstructions support convergent evolution of a low-salinity/stenohaline ancestor of a post-Paleogene arc Yucatán+Cuba *Typhlatya* clade within a euryhaline/anchialine-adapted atyid clade, and secondary adaptation of the coastal-restricted euryhaline [2-37 psu] *Typhlatya dzilamensis* (unknown conservation status), of which the remaining four species lineages are low-salinity/stenohaline adapted found in both inland and coastal regions. This study demonstrates the need for integrative/interdisciplinary approaches when conducting biodiversity assessments in complex and poorly studied aquifers.

INTRODUCTION

The Yucatán Peninsula, Mexico consists of an emerged carbonate platform spanning 165,000 km² that contains extensive karst aquifer ecosystems¹ with networks of inland and coastal caves, known as *cenotes*, the local term for sinkhole²⁻⁴. The Peninsula's unconfined aquifer has density stratification where marine origin saline groundwater mixes with the meteoric lens⁵⁻⁸. Consequently, the flooded caves are characterized by meromictic conditions with well-defined haloclines that occur progressively deeper from coastal to inland regions^{4,7,9,10}. Inland caves (Figure 1) are generally deep pit-style formations formed via collapse, whereas coastal caves are typically shallower and branch into extensive anastomotic networks^{3,10}. Biogeochemical cycling associated with the haloclines have a vital role in sustaining the subterranean food web¹¹. Collectively, the spatial variability in salinity and hydrogeology creates a collection of coastal subterranean estuaries^{5,12} that host a diverse array of characteristic stygobitic (cave-adapted) fauna¹³⁻¹⁵. These environments and associated fauna are also widely referred to as *anchialine*¹⁶⁻¹⁸. How these spatial variables shape the biodiversity and evolution of anchialine fauna remains poorly understood.

More than 170 anchialine species have been recorded in the Yucatán Peninsula, the majority of which are crustaceans^{13-15,19}. Species of the atyid shrimp genus *Typhlatya* Creaser, 1936 (Atyidae De Haan, 1849 [in De Haan, 1833-1850]) were among the first cave fauna described from the Yucatán Peninsula²⁰. The genus consists of 17 species and exhibits a globally disjunct distribution within anchialine habitats: Yucatán Peninsula, Caribbean (Puerto Rico, Dominican Republic, Lesser Antilles, Netherlands Antilles, Columbia, Honduras), West Indies (Bahamas, Turks and Caicos), Bermuda, Ascension Island, Mediterranean (France, Spain), and the Galapagos Islands; with one undescribed species from Zanzibar^{21,22}. The placement of *Typhlatya arfeae* Jaume & Bréhier, 2005, *Typhlatya galapagensis* Monod & Cals, 1970, *Typhlatya miravetensis* Sanz &

97 Platvoet, 1995, and *Typhlatya monae* Chace, 1954 within *Typhlatya* is debated, as recent
98 phylogenetic work suggests these species are more closely related to *Antecaridina* Edmondson,
99 1954, *Halocaridina* Holthuis, 1963, *Stygiocaris* Holthuis, 1960, and/or *Typhlopatsa* Holthuis,
100 1956; resulting in the paraphyly of *Typhlatya*^{21,22}.

101 To date, four species of *Typhlatya* have been described from the Yucatán Peninsula: *Typhlatya*
102 *pearsei* Creaser, 1936, *Typhlatya campecheae* H.H. Hobbs III & H.H. Hobbs Jr., 1976, *Typhlatya*
103 *mitchelli* H.H. Hobbs III & H.H. Hobbs Jr., 1976, and *Typhlatya dzilamensis* Alvarez, Iliffe &
104 Villalobos, 2005. Three of the four species are federally listed as vulnerable (*T. pearsei*, *T.*
105 *mitchelli*) or endangered (*T. campecheae*) to extinction²³. Traditional species descriptions
106 primarily utilized morphological differentiation, illustrations, and locality data as diagnostic^{20,24,25}.
107 While each species is currently accepted as morphologically distinguishable (Figure 2), character
108 variability has been reported in the case of *T. pearsei*²⁶. Because these records came from a time
109 before molecular techniques were easily accessible and widely used as standard practice, no
110 molecular data is available for type species. The lack of molecular data from type material, the
111 potential for cryptic speciation, and variable morphology poses challenges in consistent
112 identifications of subterranean crustacean species²⁷⁻²⁹.

113 In recent years, many studies have sought to understand the evolutionary history and relationships
114 within *Typhlatya* and its closest relatives^{21,22,29-34}. These investigations have produced useful
115 genetic data (mitochondrial and nuclear genes) for Yucatán Peninsula *Typhlatya* species and made
116 available via GenBank³⁵. However, it is not uncommon to find that publicly available sequences
117 have erroneous identifications leading to taxonomic and systematic confusion³⁶⁻⁴⁰. What prompted
118 the current study was an evaluation of data from previous work revealed conflicting species
119 identifications and incongruent phylogenetic hypothesis^{21,22,29-34}. Therefore, the objective of this

study was to conduct the first integrative biodiversity assessment within the poorly studied karst aquifer of the Yucatán Peninsula, through the evaluation of molecular (published and newly sequenced), morphological, broad geographic and key type localities, and environmental/salinity data, to clarify species identities and evaluate the evolution and biogeographic history of the Yucatán clade.

RESULTS

The phylogenetic/genotypic relationships of *Typhlatya* specimens were inferred from 16S rRNA (16S; 74 sequences, 515 bp), Cytochrome c oxidase subunit I (COI; 32 sequences, 1,233 bp), Cytochrome b (CYTB; 50 sequences, 588 bp), 18S rRNA (18S; 15 sequences, 1,779 bp), 28S rRNA (28S; 21 sequences, 1,090 bp) and Histone 3 (H3, 21 sequences, 327 bp). Maximum Likelihood (ML) and Bayesian Inference (BI) reconstructions based a concatenated multi-gene (select 16S, COI, CYTB, 18S, 28S and H3; 17 sequences, 5,514 bp) dataset supported two main clades and five genetic lineages (Figures 3A), represented by *T. pearsei*, *T. mitchelli*, *T. dzilamensis*, and two unknown lineages, hereafter, referred to as *Typhlatya* sp. A and *Typhlatya* sp. B; details below. Inter-clade genetic uncorrected pairwise (p)-distances of 16S and COI, ranged 5.40–18.1% and 10.0–19.5%, respectively. Intra-clade uncorrected p-distances were highest in COI for *T. dzilamensis* (1.7%) and in 16S for *Typhlatya* sp. A (1.0%), and less than 1% for remaining clades in both genes. See STable1 for summary of intra- and inter-clade uncorrected p-distances.

Phylogeny of the Yucatán Clade

ML and BI analyses of six concatenated genes (16S, COI, CYTB, 28S, 18S, H3) recovered two main clades within *Typhlatya*, each with fully supported subclades (ML Bootstrap, BS 100;

Bayesian Posterior Probability, BPP 1), and non-conflicting topologies between analyses: Clade I, *Typhlatya* sp. A + (*T. mitchelli* + *T. pearsei*) and Clade II, *T. dzilamensis* + *Typhlatya* sp. B, respectively (Figure 3A). Individuals (C1-3; n=3) sampled from the type locality of *T. campecheae* (Grutas de Xtacumbilxunam, Campeche State (CS)) were recovered within the *T. pearsei* clade. *Typhlatya* sp. A was represented by individuals sampled from Cenotes Nah-Yah (Yucatán State (YS); n=1) and Kankirixche (YS; n=1) and recovered as sister to *T. pearsei* + *T. mitchelli*. *Typhlatya* sp. B sampled from Cenote Hochtún (YS; n=1) was recovered as sister to *T. dzilamensis*. The *Typhlatya dzilamensis* clade was comprised of an individual (E4553) from the type locality (Dzilam de Bravo, YS) of this species, as well as individuals (n=3) from Cenotes Actun Ha (Quintana Roo State, QRS), Sabtun 1 (YS), and X'tabay (YS). All other species locales are provided in STable 2.

ML and BI analyses of 16S recovered congruent topologies to the multi-gene hypothesis, except for the outgroup taxon *T. garciai*, placing as sister to Clade II (*T. dzilamensis* + *Typhlatya* sp. B) in the BI analysis, however, with low support (BPP 0.67). Like the multi-gene phylogeny (Figure 3B), two main clades were recovered: Clade I (BS 85, BPP 0.99), *Typhlatya* sp. A + (*T. mitchelli* + *T. pearsei*; BS 70, BPP 0.98), and Clade II (BS 97, BPP 1), *T. dzilamensis* + *Typhlatya* sp. B.

Single-gene analyses of 28S, COI, and H3 recovered congruent topologies with the multi-gene hypothesis, although *Typhlatya* sp. B was absent from 28S and H3 analyses (SFigures 1–3). Genes 18S and CYTB recovered incongruent topologies, with *Typhlatya* sp. A absent from 18S analyses (SFigures 1–3).

Genetic (Mis)Identities of Typhlatya

The phylogenetic hypothesis resulting from the analysis of 16S (Figure 3B), including published and newly sequenced data, was incongruent with previous hypotheses and presented conflicts in species identification, except for Botello *et al.*²¹ (in part); see Figure 3C and STable 3. GenBank sequences identified as *T. mitchelli* (n=19) were consistent, except for one (KX844712²²), which was recovered here as *Typhlatya* sp. B. GenBank sequences attributed to *T. pearsei* (n=6) were consistent in identity, except for three sequences (AY115537–AY115539^{30,34}), which instead were nested within the *T. dzilamensis* clade. GenBank sequences identified as *Typhlatya* sp. (n=7) were recovered (and revised) as followed: *T. mitchelli* clade, FN995395³¹; *T. pearsei* clade, AY115540–AY115544^{30,34}; and *T. dzilamensis* clade, FN995396³¹. Lastly, individuals sampled from the type locality of *T. campecheae* (Grutas de Xtacumbilxunam; n=3) were recovered within the *T. pearsei* clade, while individuals sampled from the type locality (previously^{21,22} and in this study) of *T. dzilamensis* (Dzilam de Bravo; n=5) were recovered within the *T. dzilamensis* clade.

Single gene (i.e., 18S, 28S, COI, CYTB, H3) analyses (SFigures 1–3) resulted in similar identification conflicts as noted above, except for 18S. In total, five misidentifications were uncovered from GenBank data (summarized in Figure 3C, STable 3): i) *Typhlatya* sp. (*sensu* Hunter *et al.*³⁰ – CYTB, 16S), here recovered as *T. pearsei*; ii) *T. pearsei* (*sensu* Hunter *et al.*³⁰ – CYTB, COI, 16S; Zakšek *et al.*²⁹–28S), here recovered as *T. dzilamensis*; iii) *Typhlatya* sp. (*sensu* von Rintelen *et al.*³¹–16S, H3, 28S), here recovered as *T. mitchelli*; iv) *Typhlatya* sp. (*sensu* von Rintelen *et al.*³¹–16S, H3, 28S), here recovered as *T. dzilamensis*; and v) *T. mitchelli* (*sensu* Jurado-Rivera *et al.*²²– CYTB, COI, 16S), here recovered as *Typhlatya* sp. B. Each clade showed conflicting identities, but the highest number was found for *T. dzilamensis* and *T. pearsei*.

Species Distributions

186 Evaluation of the genetic identities of *Typhlatya* specimens using 16S and multi-gene phylogenetic
 187 approaches supported broad ranges for each of the five identified species lineages among *cenotes*.
 188 A total of 85 *Typhlatya* individuals were observed within 30 inland and coastal *cenotes* from
 189 variable salinities [0.5 – 37 practical salinity unit, psu; STable 2], here also including reassigned
 190 GenBank sequence data (Figure 4, STables 3). *Typhlatya mitchelli* (n=34; *cenotes*=12) was
 191 observed inland (YS), as well as in coastal *cenotes* (QRS). This species was sampled from ‘low
 192 salinity’ waters [<4.99 psu], above the halocline, within Cenotes Jailhouse [2.9 psu; OBH, QRS]
 193 and Tza Itza [0.63 psu; YS]. *Typhlatya pearsei* (n=14; *cenotes*=7) was found inland (YS, CS) and
 194 in coastal (QRS) *cenotes*. This species was sampled from ‘low salinity’ waters, above the
 195 halocline, within Cenotes Jailhouse [3.5 psu; OBH, QRS] and Noh-Mozon [0.7 psu; YS], and the
 196 type locality of *T. campecheae* (Grutas de Xtacumbilxunam, CS). *Typhlatya dzilamensis* (n=30;
 197 *cenotes*=11) was observed in the north/north-western coastal YS and QRS *cenotes*, spanning ~500
 198 km. This species was recorded from Cenote Sabtun 1 (YS) and Cenote Santa Maria (YS), with the
 199 greatest distance from the coastline of ~17–18 km inland. *Typhlatya dzilamensis* was the only
 200 species sampled from both ‘low salinity’ and ‘high salinity’ [>5 psu] waters [ranging 2–37 psu],
 201 above and below halocline(s). Within the OBH system (QRS), *T. dzilamensis* was observed from
 202 multiple salinities, above [2–4 psu] and below halocline(s) [33 psu], while in Cenote Crustacea
 203 (QRS), below multiple haloclines, ranging 13–34 psu. *Typhlatya* sp. A (n=4; *cenotes*=3) was
 204 observed within inland (Kankirixche and Nah-Yah, YS) and coastal [Bang, 2.5 psu; OBH, QRS]
 205 *cenotes*, sampled oligohaline waters, above the halocline. *Typhlatya* sp. B (n=3; *cenotes*=3) was
 206 also found within inland (Cenote Hoctun, YS) and coastal [4.4 psu; Na’ach Wennen Ha, OBH,
 207 QRS; and Bang, OBH, QRS] systems, sampled from low salinity waters, above the halocline.

Representatives of all five lineages were collected from the OBH cave system in waters of varying salinity (Figure 4). Specifically, species were collected sympatrically from Cenotes Bang (*Typhlatya* sp. A, *Typhlatya* sp. B, *T. dzilamensis*; ~2 psu), Jailhouse (*T. mitchelli*, *T. pearsei*; ~3 psu), and Na'ach Wennen Ha (*Typhlatya* sp. B, *T. dzilamensis*; ~4 psu), respectively. *Typhlatya* species also occurred sympatrically within Cenotes Actun Ha (*T. dzilamensis*, *T. mitchelli*; Tulum, QRS), Hoctún (*Typhlatya* sp. B, *T. mitchelli*; Hoctún, YS), and Sistema Paamul (*T. mitchelli*, *T. pearsei*; Paamul, QRS;), respectively (Figure 4).

Morphology

Typhlatya species were examined using the key developed by Alvarez *et al.*²⁵ (Figure 2; SFigure 4). *Typhlatya pearsei* and *T. campecheae* were primarily distinguished by the length of their rostrum relative to antennular segments²⁵; however, both *T. pearsei* and individuals from the type locality of *T. campecheae* exhibit a variable rostrum length extending past the eyestalk and extending towards the end of the first or midway to the second antennular segment (SFigure 4 A, B). The rostrum was found to be shorter than the eyestalk in *T. mitchelli*, whereas the rostrum of *T. dzilamensis* extends to and just beyond the eyestalk. The rostrum curves upward in *T. mitchelli*, but not in *T. dzilamensis*, consistent with species comparisons by Alvarez *et al.*²⁵. Like *T. mitchelli*, the rostrum of *Typhlatya* sp. A does not extend beyond the eyes; however, is distinct in that it appears less robust and flatter than the former. *Typhlatya* sp. B was not available for morphological evaluation. A provisional key based on genetic identification and morphological evaluation stemming from this study (and thus here excludes *T. campecheae*, see details in Discussion) can be found in Supplementary Information.

230

231 *Divergence Dating and Ancestral Reconstruction*

232 Divergence dating estimates based on a relaxed molecular clock (4 partitions: COI+CYTB, 16S,
233 28S+18S, H3; Calibrated Yule Model; Mean Log Likelihood = -50544.7; see Figure 5; Table 1;
234 STable 4) and four geological events as calibration points, recovered the genus *Typhlatya*
235 paraphyletic, with the Yucatán clade [18–39 Mya] falling within a *Cenozoic* clade [33–69 Mya].
236 The *Cenozoic* clade was comprised of *Typhlatya* species from Zanzibar, Ascension Island,
237 Bermuda, Cuba, and Yucatán Peninsula. Alternatively, SFigure 5 shows *Typhlatya garciai* from
238 Caicos Islands is included within the *Cenozoic* clade, but excluded in the primary analysis due to
239 limited/missing data. The main Yucatán clades (I and II) were each estimated to have diverged
240 10–25 Mya (Clade I, *T. mitchelli*, *T. pearsei*, *Typhlatya* sp. A) and 8–25 Mya (Clade II, *T.*
241 *dzilamensis*, *Typhlatya* sp. B), respectively. *Typhlatya mitchelli* + *T. pearsei* were estimated to
242 have diverged 4–15 Mya. A Cuban clade comprised of *T. garciai* + (*T. consobrina* + *T. taina*), and
243 recovered as sister to the Yucatán clade, with an estimated divergence of 24–49 Mya, with the
244 subclade *T. consobrina* + *T. taina* diverging 5–7 Mya. Sister to the Cuba + Yucatán clade, were *T.*
245 *rogersi* + *T. iliffei* [2–11 Mya] from Ascension Island and Bermuda, respectively, and diverging
246 32–64 Mya. *Typhlatya* sp. from Zanzibar was the first diverging lineage of the *Cenozoic* clade.
247 Results of alternative partition schemes, clock models and comparison to divergence estimates
248 from previous work^{22,23} are also summarized in STable 4B.

249 Sister to the *Cenozoic* clade included the geographically disjunct members of the *Typhlatya* /
250 *Stygiocaris* / *Typhlopatsa* (TST) complex (sensu Sanz and Platvoet⁴¹; 35–72 Mya): *T. miraventsis*
251 (Spain) + *T. arfeae* (France) [4–20 Mya], sister to a clade [29–67 Mya] including *T. monae*
252 (Dominican Republic) + (*Typhlopatsa pauliani* (Madagascar; 15–43 Mya) + *Stygiocaris*

(Australia; 8–10 Mya). The phylogeny also recovered *T. galapagensis* (Santa Cruz + Isabella Islands; 5–12 Mya) as sister group to the TST clade [48–96 Mya], albeit with low support. The outgroup *Halocaridinides* + *Halocaridina* (Hawaii; ~3 Mya) were poorly supported as sister taxa (30–90 Mya).

Within stochastic mapping, neither equal nor unequal rates models were found to be more optimal (AIC values 27.1 and 27.9, respectively) and therefore the simplest model (equal) was selected. Within the TST clade, seven of the 17 lineages are found within high salinity waters (red lineages). The high salinity lineages are interspersed throughout the TST clade, suggesting that multiple salinity transitions have occurred, although the deeper ancestral nodes within the clade remain unresolved. However, *Stygiocaris* spp. and the Yucatán+Cuba clade were each recovered with high posterior probability of low salinity/stenohaline ancestors. An independent salinity transition (i.e., *Typhlatya dzilamensis*, low to high salinity/euryhaline) was recovered within the Yucatán+Cuba clade.

DISCUSSION

On the identity “crisis” of Typhlatya within the Yucatán Peninsula

This study is the first to phylogenetically evaluate both previously published sequences and newly sequenced data across six mitochondrial and nuclear genes, along with morphological, locality, and salinity data, for *Typhlatya* species sampled across the Yucatán Peninsula. Previous studies collected new molecular data without evaluating previously published data in their assessments, resulting in phylogenetic and taxonomic conflict^{21,22,31}. A major source of confusion stemmed from the identification of *T. pearsei* and *T. dzilamensis*, given the assumed broad distribution of *T.*

275 *pearsei* (also recently reported from fresh and saline waters¹⁵) and the unknown salinity tolerances
276 of *T. dzilamensis*. Studies that correctly identified *T. dzilamensis* were sampled from the type
277 locality, Dzilam de Bravo (YS)^{21,22}. In contrast, *T. dzilamensis* specimens misidentified as *T.*
278 *pearsei* or *Typhlatya* sp. were sampled from cenotes outside of the type locality (Dzilam de Bravo)
279 and where salinities were not reported²⁹⁻³¹. The Dzilam de Bravo system is one of the few
280 scientifically documented cave system in the northern coastline (YS)^{13-15,25}, contrasting the more
281 than 1,000 km² of coastal cave passages that have been explored and documented along the
282 northeastern edge of the Peninsula (QRS)^{15,42,43}. Because of the distance between these coastal
283 systems (>200 km), previous work may not have considered the presence of *T. dzilamensis* in
284 regions well outside of its previously known range or euryhaline tendencies, leading to
285 identification errors. However, recent reports⁴³ and this study, confirms *T. dzilamensis* as a salinity
286 generalist able to traverse a vast range of salinities [2–37 psu], bringing caution to species
287 identification solely based upon cave locality and/or sampling environment⁴⁴.

288 Traditional taxonomy of *Typhlatya* species within the Yucatán Peninsula used rostrum length
289 relative to the margin of the eyes, as a critical diagnostic character (Figure 2)^{24,25,41}. Rostrum length
290 is a defining character distinguishing *T. campecheae* and *T. pearsei*²⁵, both with a rostrum reaching
291 beyond the margin of the eyes. In the original description²⁰, *T. pearsei* is described as having a
292 “short” rostrum. However, the redescription²⁴ reports variable rostrum length, with the rostrum
293 extending beyond the first antennular segment of the peduncle (proximal base of antennule)
294 somewhere between halfway through the second or just beyond the third antennular segment. This
295 variability was based upon locality, as specimens collected from re-designated type localities Gruta
296 de Chac (YS), Cenote de Hochtún (YS), and Cenote Abejas (QRS)²⁴ were reported to have a more
297 extended rostrum than other locales. In contrast, *T. campecheae* is only known from the type

locality, Grutas de Xtacumbilxunam (CS), and reported with a rostrum length reaching halfway or through the first antennular segment of the peduncle^{24,25}. Within this study, the inclusion of individuals sampled from Grutas de Xtacumbilxunam were genetically recovered within the *T. pearsei* clade (Figure 3A) and were not distinguishable by rostrum length from *T. pearsei* (both exhibited rostral length to the first segment and beyond) (SFigure 4 A, B). Therefore, *T. campecheae* is identified as a phenotypic variant of *T. pearsei* and should be considered a junior synonym, given taxonomic priority²⁰. Variation of rostrum length within species has been reported in other members of Decapoda, such as the stygobitic genus *Troglocaris* or species *Aristeus antennatus*⁴⁵⁻⁴⁶. The type locality of *T. pearsei*, Cenote Balankanché (X Calakooop, YS), is a restricted access archaeological site and tourist attraction, thus specimens were not available for additional comparisons. A combined morphological and molecular investigation of *T. pearsei* will be needed to properly address this issue.

In addition to clarifying the identity of known species within the Yucatán Peninsula, two genetic lineages were newly revealed in our analyses, adding to the current taxonomic complexity (Figure 3 A–B). *Typhlatya* sp. A was recovered as sister to *T. mitchelli* + *T. pearsei* and was not recovered among GenBank data (Figure 3). Like *T. mitchelli*, *Typhlatya* sp. A has a short rostrum that does not extend beyond the margin of the eyes. However, the distal edge of the rostrum of *Typhlatya* sp. A appears thinner and less robust than that of *T. mitchelli* (SFigure 4 C, E). *Typhlatya* sp. B was recovered as sister to *T. dzilamensis*, with one sequence previously misidentified as *T. mitchelli* (KX844712²²). Unfortunately, voucher material for *Typhlatya* sp. B was not available for morphological evaluation, as subsampled specimens were used for molecular analyses. It should be noted that these new lineages came to light post-genetic evaluation and based on few samples (n≤4 each).

Another unexpected result was the sympatry among all five lineages within low salinity waters within the OBH system. Other caves also harbored at least two species (i.e., Actun Ha, Hochtún, Sistema Paamul). Thus, caution should be applied to species identification based upon morphology and salinity preferences alone.

Salinity Tolerance of Anchialine Atyidae

Atyidae is a diverse, globally distributed family of predominantly freshwater shrimp species that includes several independent clades of stygobitic taxa, and at least one identified as *anchialine*^{31,48}. So far, atyid species have not been reported from ocean environments^{31,48}. The adaptation for increased salinity tolerance is found across multiple lineages within the anchialine clade comprised of *Halocaridinides* + *Halocaridina* + TST complex^{22,31,41}. While the reconstruction of ancestral salinity preferences for this anchialine clade (and the TST complex within) was unresolved, the ancestor of the Yucatán+Cuba clade was recovered as having a low salinity preference. All descendants within the clade inhabit low salinity environments, except for euryhaline *T. dzilamensis*, suggesting that *T. dzilamensis* historically transitioned from low to high salinity. *Typhlatya garciai* was originally reported as a freshwater species from Cuba⁴⁹; however, later recorded from brackish/saline cave waters of Caicos Islands⁵⁰. Representatives of *T. garciai* from both locations were not recovered as monophyletic in our analyses, and the inclusion of *T. garciai* from Caicos in the supplemental ancestral reconstruction (SFigure 5) also recovered a single transition (*T. dzilamensis*) to a low salinity ancestor for the Yucatán+Cuba clade.

Understanding the evolution of salinity tolerance within anchialine fauna is critical regarding historic sea-level change. Sea-level fluctuations impact underlying aquifers, where micropaleontological observations of benthic meiofauna drastically change with hydrogeology (e.g., fresh↔anchialine↔marine) over time^{16,51-54}. Depending upon the salinity tolerance of an

anchialine species, sea-level fluctuation can likely result in significant changes to its respective range and distribution⁵⁴. Subsequently, periods of isolation and expansion within a karst aquifer the may potentially acting as a driver of diversification within anchialine fauna.

Evolution within crevicular spaces

How anchialine fauna have achieved such disjunct distribution patterns despite inhabiting seemingly isolated environments remains a significant biogeographical question. *Typhlatya* species are interspersed throughout the Caribbean, West Indies, Bermuda, Yucatán Peninsula and in the West Atlantic^{21,22}. However, the Yucatán Peninsula is of particular interest, as it hosts the greatest known diversity (five lineages) of *Typhlatya* in the world. Previous divergence estimates for the split of the Yucatán clade from the Cuban clade has ranged considerably, 14–27 Mya²¹ to over 74 Mya²² (Table 1; STable 4). In the present study, this split was estimated to be 23–49 Mya (Figure 5; Table 1; STable 4). The disparate age ranges at this node are likely due to the use of different time-calibrations in the molecular clock analyses, data types (mitochondrial genomes vs. gene fragments) and inclusion of different taxonomic representatives. The present study and Botello *et al.*²¹ utilized recent biogeographic events, while Jurado-Rivera *et al.*²² prioritized deep fossil calibrations outside the anchialine atyid clade. Therefore, the discussion below is in consideration of the findings of the current study.

The Yucatán Peninsula and the Paleogene Arc (Cuba+Hispaniola) are hypothesized to have been connected via a land span in the Paleocene through Middle Eocene (until ~49 Mya) and subsequently split after the formation of the Cayman Trough⁵⁵⁻⁵⁷. What has been termed the “Paleogene arc drift vicariance scenario” may explain the disjunct distribution patterns of modern-day taxa, such as those of certain fish (Cichlidae)⁵⁷. This vicariant event may coincide with divergence times estimated for the split of the Yucatán+Cuba clade [24–49 Mya]. *Typhlatya*

species (and anchialine atyids) have not been reported outside of groundwater aquifers, and yet open ocean dispersal to isolated oceanic islands (e.g., Bermuda) has been proposed^{30,58}. In contrast, this study suggests that dispersal opportunities via spelean corridors through established (or temporary) tectonic and/or volcanic connections are plausible^{30,59,60} and likely dependent on the availability of suitable anchialine habitat and influenced by historic sea-level change^{16,51-54}. Therefore, the low salinity/stenohaline ancestor, as proposed here, of Yucatán+Cuba *Typhlatya* may have migrated along the Paleogene arc via spelean corridors established between Cuba⁶¹ and emerged portions of the Yucatán Peninsula⁶², and where the meteoric lens would have expanded during periods of low sea-level^{16,51-54} until the eventual split of these landmasses post formation of the Cayman Trough.

The Yucatán platform was predominantly submerged during the Cretaceous, with global sea-level oscillations leading periods of partial submergence and emergence since its formation^{51-54,61-63}. The ancestor of the Yucatán clade likely invaded an area that was not the fully emerged Peninsula observed today. Rather, depending on the time of colonization, the ancestor could have potentially found suitable anchialine karst habitat along historic coastlines, such as the southern Yucatán Peninsula. The high concentration of inland *cenotes* northeast of the La Libertad arch (CS)¹, may have also offered historic low salinity refuge (and means of expansion) during sea-level change. Thus, species likely followed the expansion and retraction of the meteoric lens vertically within the Yucatán platform. Yucatán freshwater cyclopinid copepods are hypothesized to have followed a similar biogeographic pattern, with southern populations migrating northward and experiencing isolation events post-Pliocene^{64,65}, however divergence times for *Typhlatya* species are likely due to older events [4-25 Mya]. Vertebrates, such as the cave dwelling freshwater catfish, *Rhamdia*

guatemalensis (Günther, 1864), show patterns consistent with colonization from the southernmost region of the northern Peninsula and subsequently expanded north and north-eastward^{66,67}.

The precise timing and events leading to the diversification of Yucatán *Typhlatya* and transition to euryhaline in *T. dzilamensis* is difficult to discern. This study, however, reveals that present-day distributions appears to be influenced by salinity and hydrogeology. The ability of *T. dzilamensis* to cross the salinity barrier offers a great advantage to expand ecological niches and access a broader range of carbon and energy sources than previously estimated, when compared to its stenohaline counterparts⁴⁴. *Typhlatya dzilamensis* was observed above and below the halocline within OBH (QRS) and Crustacea (QRS), and sympatric in the meteoric lens with all four species within the same system of cenotes (OBH) only a few kilometers apart, and with *T. mitchelli* in Actun Ha (Tulum, QRS). *Typhlatya dzilamensis* also has an extensive range from at least the north/northwestern and eastern coastal margins (> 500 km); whereas *T. pearsei*, *T. mitchelli*, *Typhlatya* sp. A, and *Typhlatya* sp. B are spatially restricted to the meteoric lens of inland (YS) and coastal cenotes (QRS), ~200 km in distance (Figure 4A). The genetic connectivity among Yucatán populations has been explored within *T. mitchelli*³⁰, where the analysis of the fast-evolving CYTB revealed low haplotypic diversity over distances of up to 235 km. Although haplotype diversity was not measured in the current study, intra-clade pairwise genetic distances for *T. mitchelli*, *T. pearsei* and *T. dzilamensis* were generally less than 1% for 16S and COI, respectively, thus supporting broad dispersal capabilities throughout inter-connected spelean networks of the aquifer.

Implications for Continuing Integrative Research

Biodiversity management and conservation of karst aquifers⁶⁸ need more studies with an interdisciplinary research framework via the collection of integrative data (e.g., phylogeny,

morphology, DNA barcoding, hydrogeology/water quality, biogeochemistry, paleoecology, etc.)^{69,70}. This study serves as a case study to provide guidance (and caution) for continuing research on poorly studied stygobitic biodiversity of the Yucatán aquifer.

The current findings bring forth the need for revision and re-evaluation of the conservation status of *Typhlatya* species within the Yucatán Peninsula. The Official Mexican Standards for Environmental Protection (NOM-059-SEMARNAT-2010)²³ lists three of four known species: *T. campecheae* as federally endangered, and *T. mitchelli* and *T. pearsei* as federally threatened, while IUCN Red List classifies these species as *stable* and of *least concern*⁷¹. The results unambiguously showed that *T. campecheae* is likely not endemic to Grutas de Xtacumbilxunam (CS), nor a distinct species from the *vulnerable* *T. pearsei*; thus, the *endangered* status should be re-evaluated. Instead, our findings support an expansive distribution of *T. pearsei* within the meteoric lens of inland (YS and CS) to coastal *cenotes* (QRS), with the *vulnerable* *T. mitchelli* having an overlapping range; not recorded from CS *cenotes* (to date). *Typhlatya dzilamensis* is not federally listed and yet was here found to be distinct from all other species, representing the only high salinity species with a habitat range restricted to coastal systems throughout the Peninsula. Thus, *T. dzilamensis* is an excellent candidate in need of evaluation, given its association with karst coastlines, which are particularly vulnerable to anthropogenic impacts and coastal development changes^{68,72,73}. Lastly, the identification of two unknown species, which were ‘rare’ among our newly sequenced samples and in previously published data, warrants further study to better understand their distributions and population genetic structure. Pending species descriptions, they are likely candidates for elevated conservation status.

The presence of *Typhlatya* within inland and coastal *cenotes* throughout the Peninsula, and their strict affinity to particular salinities and hydrogeological constraints, identifies member species as

potential models to assess groundwater health. Given temporal high energy meteorological events (e.g., hurricanes)⁷⁴, and other environmental impacts^{67,71,72}, prevalent in Yucatán region and continuing sea-level rise⁵⁸⁻⁶⁰, *Typhlatya* species can serve as biological indicators^{75,76} and/or indicators of ecological resilience⁷⁷ to environmental change. The phylogeny presented here provides a robust evolutionary hypothesis for conducting comparative studies among closely related species to understand adaptation and key functional roles⁷⁰. The presence of a salinity generalist within coastal cave systems, like *T. dzilamensis*, provides an excellent opportunity to evaluate the functional role of this adaptation in carbon-energy transfer from the meteoric lens (where organic matter is abundant) to the extremely oligotrophic saline groundwater¹¹.

The aforementioned would be challenging without discrete species clarity⁷⁸. Therefore, the *Typhlatya* research presented here can serve as a baseline for integrative and multi-disciplinary investigations with local experts and researchers, to provide robust scientific evidence to support policy, conservation efforts and biodiversity assessments of the extensive karst aquifer ecosystems within the Yucatán Peninsula.

METHODS

Taxon sampling

Forty-one individuals within the genus *Typhlatya* were collected during several field expeditions to caves throughout the Yucatán Peninsula between 2013 and 2019 (See Figure 4A; STable 2, 3). Efforts were taken to collect representatives from type localities to address contentious identifications, namely *T. dzilamensis* from Cenotes Buya-Uno, Cervera, and Dzilamway (Dzilam de Bravo, YS) and *T. campecheae* from Grutas de Xtacumbilxunam (CS). Samples from the type

locality (Grutas de Balankanche) of *T. pearsei* were not obtained for this study due to its status as a protected archaeological site. Initial morphological identification of specimens (except from specimens sampled below, OBH) followed Alvarez *et al.*²⁵. Unfortunately, access to type material located at the Smithsonian Institution National Museum of Natural History is not currently possible due to COVID-19 restrictions.

Additionally, collections of *Typhlatya* were conducted from four caves (Na'ach Wennen Ha, Odyssey, Jailhouse, Bang) within the OBH system (QRS) along a geographic transect from the Caribbean coast (Figures 3B, 3C), as first described by Alvarez *et al.*²⁵. Specimens were also collected from Cenote Crustacea (QRS) and the Dzilam de Bravo (type locality of *T. dzilamensis*). Specimens from these sites were collected “blindly” (i.e., without identifying to species prior to preservation or sequencing) only making note of the environmental conditions (salinity, temperature, depth) within cave sections of specimen collection. Individuals were collected by cave divers using sampling bottles. Sampling depth and salinity were measured when possible, using either YSI XLM-600, EXO-02, Hydrolab DSS, or SonTek CastAway CTD devices. Salinity concentrations were classified as followed: Low salinity [0.5–4.9 psu], oligohaline meteoric lens and generally above primary halocline, and High salinity [5–37 psu], mesohaline to polyhaline groundwater and generally below the primary halocline; although, we recognize that these are not discrete and some species may exhibit overlap between salinity designation (e.g., *T. dzilamensis*). These designations are based on collection of specimens of this study and simplified from previous salinity classifications⁷⁹. The primary halocline is recognized as the mixing zone below the meteoric lens, where salinity levels can be mesohaline (e.g., Casa Cenote, near Tulum, QRS) to euhaline (>30 psu; e.g., Giant Cave, Belize). However, salinities above five psu were not observed within the meteoric lens of collection sites in this study. Salinities below the halocline were

variable (and may include multiple haloclines), ranging from mesohaline (5–8 psu)⁷⁹ to saline (>30 psu)⁷⁹. Specimens and associated salinities are indicated in Figure 3B and STable 2. Within six hours of collection from the OBH transect, specimens were wrapped and stored at 0 °C in prebaked (450 °C for 4 h) aluminum foil. The specimens were transported frozen on dry ice, and then stored in the laboratory at –20 °C. While frozen, select specimens were subsampled (i.e., abdomen) for molecular analysis and transferred to 95–100% ethanol, with the remaining portion being used for stable isotope and fatty acid analyses¹¹. Other collected material was fixed in ethanol (90–100%) immediately, or frozen with liquid Nitrogen or dry ice, and stored at -20°C or -80°C until ready for DNA extraction. Morphological and molecular vouchers are deposited at the Collection of Crustacea, UNAM-Sisal, Yucatan, MX (XXXXXX–XXXXXX).

GenBank Selection, DNA Extraction

All available mitochondrial and nuclear gene sequences for *Typhlatya* species (*T. pearsei*, *T. mitchelli*, *T. dzilamensis*, and *Typhlatya* sp.) from the Yucatán Peninsula were downloaded from GenBank (n=112; STable 3). This dataset included gene fragments of mitochondrial 16S rRNA, COI (two partial fragments) and CYTB, and nuclear H3, 18S rRNA, and 28S rRNA (STable 3). Of all genes, 16S rRNA was one of the most consistently utilized gene across separate studies and therefore represents the broadest sampling used for the phylogenetic evaluations presented here. Multi-gene analyses included select individuals as clade representatives. Individual genes trees of COI, CYTB, H3, 18S rRNA, and 28S rRNA are provided within the supplemental (Figs. S1–5).

Tissue was removed from 41 individuals and DNA was extracted following standard protocols of either ethanol precipitation or the Qiagen DNeasy Tissue and Blood kit. Genes 16S rRNA, COI, CYTB, H3, 18S rRNA, and 28S rRNA were amplified using primers outlined in STable 5. PCR

mixtures consisted of DNA template (1-2 µL), forward primer (1 µL), reverse primer (1 µL), water (9.5 µL), and GoTaq® Green Mastermix (12.5 µL). PCR temperature profiles are specific to each primer and were run using the BIORAD T100 Thermocycler. Successful amplification was observed via gel electrophoresis and purified with ExoSAP-IT PCR Product Cleanup kit. Cleaned PCR products were then sent for sequencing at the Texas A&M Corpus Christi Genomics Core Lab. Geneious Prime 2020 v.2.3–2021 v.0.1⁸⁰ was used to examine, clean, and assemble raw sequence data. Protein-coding genes were checked for the presence of stop-codons and pseudogenes and all sequences were checked for contamination via NCBI Blast. Newly generated gene data (16S=40; COI=8; CYTB=11; 18S=11; 28S=13; H3=14) were subsequently submitted to GenBank (Accession Numbers: XXXXX-XXXXX) (STable 3).

Phylogenetic Analyses

The 16S rRNA dataset was compiled from both GenBank (n = 34) and newly generated data (n = 40). Sixteen of the 41 newly sequenced individuals were used for our multi-gene concatenated analyses (16S rRNA, COI, CYTB, H3, 18S rRNA, 28S rRNA; n=5514 bp). Individuals were selected based upon their sampling locality, species identification, and sequencing success of at least three of the targeted six genes. Gene alignments were concatenated using SequenceMatrix⁸¹. Sequence data for each gene were aligned via MAFFT⁸² local iterative (18S, COI, CYTB, H3) and global iterative method (16S, 28S). Phylogenetic analyses using both Maximum Likelihood (ML) and Bayesian Inference (BI) were conducted on XSEDE within the CIPRES Science Gateway⁸³. *Typhlatya consobrina* Botoşăneanu & Holthuis, 1970, *Typhlatya garciai* Chace, 1942, and *Typhlatya taina* Estrada & Gómez, 1987 were selected as outgroup taxa based upon previous phylogenetic assessments²². Different individuals from the same outgroup species (*T. taina* and *T. consobrina*) were concatenated together to provide the largest available dataset for comparison

(see STable 3). JModelTest⁸⁴ was utilized to select optimal substitution models for each gene based upon corrected AIC for BI (see STable 6). Maximum likelihood analyses were conducted using RAxML⁸⁵ and node support quantified via rapid bootstrapping with 1,000 replicates. Bayesian analyses were conducted using MrBayes v.3.2.7a⁸⁶ with 30 million generations, a burn-in of 10 million, sample frequency every 1,000 generations, and with two runs and four chains. Tracer⁸⁷ v. 1.6.0 was used to confirm convergence of individual chains and examine effective sample size (ESS>200). Analyses were visualized using FigTree⁸⁸ version 1.4.4 (Figure 3).

Divergence Dating

Divergence dating of nodes was performed using Beast⁸⁹ v.2.6.3 based on the multi-gene (16S rRNA, COI, CYTB, 18S rRNA, 28S rRNA, H3) dataset, with an expanded taxon sampling that included members of the *Typhlatya/Stygiocaris/Typhlopatsa* (TST) complex, following Jurado-Rivera *et al.*²² *Typhlatya galapagensis* (Islas Isabella / Santa Cruz), *Halocaridina rubra* (Hawaiian Islands) and *Halocaridinides fowleri/H. trigonophthalma* (concatenated) were included as outgroup taxa, with trees rooted using *H. fowleri/H. trigonophthalma* (STable 7). Divergence dating analyses used the following partitioning schemes: I) 16S + 18S / 28S + COI / CYTB + H3 or II) 16S + 18S / 28S + COI / CYTB + H3 + COI / CYTB, codon 1+2, with codon 3 excluded). Each gene partitioning scheme were analyzed by enforcing the following molecular clock parameters: i) strict (partition I); ii) strict (partition II); iii) relaxed uncorrelated lognormal distribution (partition I); and iv) relaxed uncorrelated lognormal distribution (partition II) and the Calibrated Yule speciation model (see STable 4 for additional parameter details). Each tree used the following biogeographic events as calibration points for the minimum age diversification of the following TST sister taxa, following Botello *et al.*²¹: a) isolation of *T. galapagensis* divergent populations (8.0% pairwise distance) on Isabella and Santa Cruz Islands, with the ancestor not

older than the Coco Ridge; 5-14 Mya; b) isolation of *Stygiocaris lancifera* Holthuis, 1960 and *Stygiocaris stylifera* Holthuis, 1960 ancestor post emergence of the Cape Range anticline (Australia); 7-10 Mya and c) isolation of the *T. consobrina* + *T. taina* ancestor post Havana–Matanzas Channel closure (Cuba); 5-6 Mya. Additionally, the approximate age for the diversification of *Halocaridina rubra* Hawaiian complex⁵⁶ was also included based on minimum ages of their presence on the islands (0.5–5 Mya)⁹⁰, but does not estimate the age of the origin of the species, which is much older. Site models for each partition used empirical gamma shape, proportion of invariable sites and substitution rates were estimated via BIC using JModeltest. Substitution rates were estimated and assumed independent among partitions and clock models and tree priors were linked. Two independent runs for each clock model + partition scheme were performed for 30 million generations and sampling every 1000 generations. Outputs from each independent run (.log and .trees files) were combined using LogCombiner⁸⁹ v.2.6.3, excluding the initial 10% of the burn-in, checked for convergence using Tracer⁸⁷ v.1.4.4 and trees were summarized using TreeAnnotator⁸⁹ v.2.6.3.

Stochastic Mapping of Salinity Preference

Ecological transition of salinity preference within anchialine Atyidae was reconstructed via stochastic mapping over the ultrametric tree from divergence dating analyses (above) and used phytools⁹¹, ape⁹², and geiger³² packages within R Studio⁹⁴. Salinity data was compiled from the literature and observations from the present study (see STable 2, 7). Salinity values were split into two discrete characters: (1) low salinity tolerance [0.5-5 psu] and (2) high salinity tolerance [5-37 psu]. To determine whether salinity states have transitioned at equal or unequal rates, both models were compared using the AIC. Software SIMMAP⁹⁵ was used assuming equal rates, an equal

estimation at the root, with 100 simulations on the summarized ultrametric tree. Simulations were combined to visualize the probability density of each discrete character (Figure 5).

Map Construction

The distribution of 85 individuals of *Typhlatya* within the Yucatán Peninsula was visualized using software program QGIS v.3.1⁹⁶. Of which, 44 locality records were obtained from previous studies^{21,22,29-34} and 41 records are newly generated from this study. Species identities were assigned from the single-gene phylogenies of the present study (above). Vector map data was obtained from Natural Earth (www.naturalearthdata.com). Cenotes of the type locality of *T. dzilamensis* (Dzilam de Bravo, Cervera) were treated as one locality.

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Data Accessibility

Newly obtained sequences were deposited in GenBank Repositories under accession numbers listed in STables 3 and 7. All data, including phylogenetic trees and R-scripts used throughout this study are publicly available on the Open Science Framework (OSF) repository (ID: XXX)

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Author Contributions

840 LB, DB, ECS and EB designed this study. LB, ECS, DB, FA, TI and EB contributed to the
841 collection and preservation of samples used in this study. All sequence data collection, editing and
842 analyses, as well as phylogenetic and evolutionary reconstructions were carried out by LB and EB,
843 with assistance from BG. LB designed all figures, with contributions from DB, BG and EB. LB,
844 AL, SR, LS, ECS contributed to photo-documentation and images. Funding provided by EB, TI,
845 NS, MPM and FA. LB and EB wrote the manuscript. All authors edited and approved the final
846 draft.

847

848 **Ethics Declaration**

849 The authors declare no competing interests

1 **Table 1.** Comparison of mcraAges of Yucatán and Cuba clades estimated in previous work^{21,22} to those of the current study. Results
2 are based on the relaxed, log-normal clock model with four gene partitions (COI+CYTB, 16S, 18S+28S, H3) and four geological
3 events as calibration points following Botello et al.²¹. See also STable 4 for results from all analyses.

Clade	Jurado-Rivera et al., 2017			Botello et al, 2013			Relaxed (4 part)		
	Height		95% HPD	Height		95% HPD	Height		95% HPD
	Mean	Upper	Lower	Mean	Upper	Lower	Mean	Upper	Lower
Yucatán <i>Typhlatya</i>	56.50	70.07	43.11	14.91	20.22	9.70	28.12	38.75	18.23
<i>Typhlatya</i> sp. A + <i>T. mitchelli</i> + <i>T. pearsei</i>	--	--	--	--	--	--	16.75	24.76	9.92
<i>T. mitchelli</i> + <i>T. pearsei</i>	--	--	--	4.70	6.90	2.70	9.01	14.58	4.32
<i>T. dzilamensis</i> + <i>Typhlatya</i> sp. B	33.35	44.89	22.36	--	--	--	16.34	25.39	8.19
<i>T. garciai</i> + (<i>T. taina</i> + <i>T. consobrina</i>)	--	--	--	--	--	--	30.28	42.94	18.09
<i>T. taina</i> + <i>T. consobrina</i>	43.52	58.66	29.25	5.52	5.99	5.05	6.18	7.07	5.33
Cuba + Yucatán <i>Typhlatya</i>	--	--	--	20.12	26.54	14.24	35.23	48.65	23.71
<i>T. garciai</i> + (Cuba + Yucatán <i>Typhlatya</i>)	74.01	88.61	60.08	--	--	--	--	--	--
<i>T. garciai</i> + (Yucatán <i>Typhlatya</i>)	69.29	82.62	55.40	--	--	--	--	--	--

1 **Figure 1.** Select field sites and sampling of *Typhlatya* specimens within the Yucatán Peninsula.
2 **A.** Entrance to Grutas de Xtacumbilxunam (Campeche State), showing pit-style inland *cenote*;
3 depth ~60 meters; note replica of wooden ladder used by Mayans to access aquifer; **B.** Cenote
4 Noh-Mozon (inland, Yucatán State), another inland cave, and collection site of *Typhlatya pearsei*;
5 **C.** Cave diver (ECS) collection of *Typhlatya mitchelli* within Cenote Tza Itza (inland, Yucatán
6 State) using catch net and falcon tube; **D.** Post collection (by ECS) of *Typhlatya campecheae*
7 within Grutas de Xtacumbilxunam, type locality; **E.** Cave diver (ECS) emerging from freshwater
8 entrance pool within Grutas de Xtacumbilxunam; collection site of *Typhlatya campecheae*.
9 Images: A-D, Kay Vilchis; E. Erick Sosa

1 **Figure 2.** A. Graphical morphological key of *Typhlatya* spp. of the Yucatán Peninsula adapted
2 from Alvarez *et al.*²⁵. B-E. Species of the Yucatán Peninsula. B. *Typhlatya pearsei* ('Ultimo
3 Suspiro', near Dzilam de Bravo); C. *Typhlatya campecheae* (Grutas de Xtacumbilxunam;
4 ID=C2*). D. *Typhlatya dzilamensis* (Cenote Crustacea). D. *Typhlatya mitchelli* (Sistema Paamul;
5 ID=TI008D*). a, antennular peduncle; e, eyes, r, rostrum. Images taken by ECS, AL, SR, and AS.
6 *Genetically identified in this study, Figure 3.

A

Typhlatya pearsei

Typhlatya campecheae

Typhlatya dzilamensis

Typhlatya mitchelli

Figure 3. Phylogenetic hypotheses of *Typhlatya* spp. from the Yucatán Peninsula. A. Multi-gene (16S rRNA, COI, CYTB, 18S rRNA, 28S rRNA, H3) phylogeny of select *Typhlatya* spp. sequences, Bayesian phenogram shown. B. 16S rRNA phylogeny of GenBank and sampling new to this study, Bayesian phenogram shown. C. Graphical summary of phylogenetic relationships from previous studies^{21,22,30,31}. Support values indicated at nodes above branches: Bootstrap/Bayesian Posterior Probability. Supports values <90/0.90, not shown (see text) and indicated by '-'. Colors represent species identity resulting from the molecular evaluations of the study (3A-C): *Typhlatya pearsei* = yellow, *Typhlatya mitchelli* = red, *Typhlatya dzilamensis* = blue, *Typhlatya* sp. A = purple, *Typhlatya* sp. B = orange. Filled black shapes beside species' identifications (3B) indicate the previous identifications of GenBank material that conflict with the present study: circle = *Typhlatya* sp., triangle = *T. pearsei*, square = *T. mitchelli*. Shapes beside species' identifications (3C) indicate all previous identifications from previous work: circle = *Typhlatya* sp., triangle = *T. pearsei*, square = *T. mitchelli*, star = *T. dzilamensis*. Sequences from specimens from type localities indicate by colored stars (A, B), green star = *T. campecheae*, Grutas de Xtacumbilxunam; blue star = *T. dzilamensis*, Dzilam de Bravo. Sequences from samples with known salinity (in psu) are indicated by colored water droplets (B), blue = low salinity, 0.5-4.99, red = high salinity, 5-37.

A 16S rRNA, COI, CYTB, 18S rRNA, 28S rRNA, H3

- ★ Type locality of *T. campecheae*
- ★ Type locality of *T. dzilamensis*

B 16S rRNA

- 0.5-4.9 psu
- 5-37 psu
- *Typhlatya* sp.
- ▲ *T. pearsei*
- *T. mitchelli*

C

- = *T. mitchelli*
- △ = *T. pearsei*
- = *Typhlatya* sp.
- ☆ = *T. dzilamensis*

Hunter et al., 2008
CYTB, 16S

Von Rintelen et al., 2011
16S, 28S, H3

Botello et al., 2013
16S, COI, CYTB, 18S, 28S, H3

Jurado-Rivera et al., 2017
13 protein coding mt genes

Figure 4. Geographic distribution of *Typhlatya* spp. included in this study. A. Sampling distribution of *Typhlatya* spp. (Yucatán Peninsula); B. Sampling distribution of *Typhlatya* spp. (Quintana Roo State, QRS); C. Hypothetical cave profile of the Ox Bel Ha system (QRS) and schematic sampling distribution of the five species identified in this study, collected by salinity layer. Colored symbols indicate species identification: *Typhlatya pearsei* = yellow, triangle; *Typhlatya mitchelli* = red, square; *Typhlatya dzilamensis* = blue, star; *Typhlatya* sp. A = purple, circle; *Typhlatya* sp. B = orange, circle. Cave profile adapted from Brankovits et al.¹¹. Maps were made in QGIS using metadata from Natural Earth.

Figure 5. Chronogram showing divergence estimates [in brackets; Mya, 95% high posterior density limits as confidence intervals], based on a relaxed, lognormal clock, calibrated Yule model and 4 gene partitions (COI+CYB, 16S, 18S+28S, H3) of the anchialine Atyidae clade. Stars at nodes indicate calibration points used in analyses. Stochastic mapping of salinity trait evolution is visualized with a density map overlaying the chronogram. Posterior probability (PP) of high salinity/euryhaline (state=2; 5–37 psu) is colorized, with red reflecting high PP, blue reflecting low PP (and therefore, low salinity/stenohaline; 0–4.9 psu), and purple reflecting uncertainty in SIMMAP analyses.

Supplementary Files

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