A new locality and phylogeny of the stygobitic *Typhlatya* shrimps for the Yucatan Peninsula

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Typhlatya is a genus of small, stygobitic shrimp in the family Atyidae. Species in this genus are found in subterranean anchialine habitats, and they span salt, brackish and fresh waters. None have been reported in the open sea (Botello et al. 2013). Phylogenetic studies indicate that the genus, as presently defined, is paraphyletic and needs to be redefined, as two of its 17 described species cluster with members of different genera: *Typhlatya galapagensis* Monod & Cals, 1970 clusters with the widespread *Halocaridina* and *T. monae* Chace, 1954 likely clusters with the Australian *Stygiocaris* (Botello et al. 2013; Jurado-Rivera et al. 2017).

Typhlatya species often have very small ranges, limited to single caves, islands, or portions of a coast (Botello et al. 2013). The genus has a broad distribution in many coastal subterranean habitats. Species are found in the West Mediterranean region (France and Spain), West Indian Ocean (Zanzibar), Caribbean region (the Antilles, Bahamas and Yucatán), south Atlantic sea (Ascension Island) and the Galápagos (Botello et al. 2013; Jurado-Rivera et al. 2017). Their extremely broad and disjunct distribution has been suggested to derive from both plate tectonic vicariance and dispersion coupled to a so-called 'Tethyan' distribution pattern (Buden and Felder 1977; Stock 1993; Jurado-Rivera et al. 2017). Isolation of the ancestral lineages would coincide with the fragmentation of the Tethys Sea, which was a predominantly shallow-water circumtropical ocean in the late Mesozoic Era and Tertiary Period (Sterrer 1973; Stock 1993). The collision of continental landmasses and the formation of broad, deep oceanic basins could have resulted in the allopatric diversification of the ancestors of present-day species, which subsequently became stranded in inland aquifers.

Evidence for recent dispersal is observed in several *Typhlatya* species that display relatively broad distributions separated by stretches of sea, and still maintain panmixia (Botello et al. 2013). For example, *T. monae* is known from two sides of the Caribbean: to the northeast, they inhabit Mona Island, Puerto Rico, Hispaniola (Greater Antilles) and

Barbuda (Lesser Antilles) and Curacao and the San Andres Islands to the southeast. Another example is *T. garciai* Chace, 1942 and *T. kakuki* Alvarez, Iliffe & Villalobos, 2005, known from Providenciales (Caicos Islands) and Acklins Island (Bahamas), which, despite being separated by a 173-km deepwater sea arm, have very low genetic divergence (Botello et al. 2013). This could be explained by continuous gene flow via dispersal over sea or by recent colonization and subsequent isolation (Botello et al. 2013). A high dispersal potential of *Typhlatya* across subterranean waters is also reported on the Yucatan Peninsula, where populations of *T. mitchelli* Hobbs & Hobbs, 1976 are separated by up to 235 km, yet still share haplotypes (Hunter et al. 2008).

 Four species of *Typhlatya* have been described in the Yucatan Peninsula (**Figure 1**): *T. dzilamensis* Álvarez, Iliffe and Villalobos, 2005; *T. pearsei* Creaser, 1936; *T. mitchelli*; and *T. campecheae* Hobbs & Hobbs, 1976. In addition, a *Typhlatya* sp. is known from Cenote San Antonio Chiich, for which genetic data support an independent species status for this apparent endemic population (Hunter et al. 2008). *Typhlatya pearsei* and *T. mitchelli* have an extensive and broad range in many cenotes throughout the states of Yucatan and Quintana Roo. *Typhlatya campecheae* is endemic to Grutas de Xtacumbilxunam (Hobbs and Hobbs 1976). *Typhlatya dzilamensis* inhabits saltwater and has been described as endemic to three nearby cenotes, Cenote Cervera, Cenote Dzilamway, and Cenote Buya Uno, localized near Dzilam de Bravo on the seacoast of the northern Yucatan state (Álvarez et al. 2005). The other three species inhabit freshwater.

 The purpose of this study was to analyze the phylogenic relationship of a new population of *Typhlatya* discovered from Cenote Xtabay of the Ponderosa system in the state of Quintana Roo, Mexico (20º29'57.12" N, 87º15'39.06 W), to establish which species they are most closely related. Three specimens were collected by E. Chávez on 28 October 2017 at a depth of 14 m below the halocline in saltwater and deposited in ethanol. Collection of specimens was authorized under collection permit SEMARNAT/SPGA/DGVS/02068/17.

 Genomic DNA was extracted using the Qiagen DNEasy® Blood & Tissue Kit by digesting a leg in lysis buffer from each of the three specimens. We PCR amplified and sequenced a single 328-bp fragment for the nuclear histone H3 locus using the primer pairs H3aF (5'-ATGGCTCGTACCAAGCAGACVGC-3') and H3aR (5'-ATATCCTTRGGCATRATRGTGAC-3') following standard protocols (Espinasa et al. 2007). Amplification was carried out in a 50-µl volume reaction with Qiagen's Multiplex PCR kit. The PCR program consisted of an initial denaturing step at 94 ºC for 60 sec, followed by 35 cycles (94 ºC for 15 sec, 49 ºC for 15 sec, 72 ºC for 15 sec), and a final extension at 72 ºC for 6 min in a GeneAmp® PCR System 9700 (Perkin Elmer). PCR products were then cleaned with the Qiagen QIAquick PCR Purification Kit and sent to SeqWright, Huston, Texas for Sanger sequencing. Resulting chromatograms were trimmed and contigs created using the sequence editing software SequencherTM 3.0. External primers were excluded. Sequence identity was confirmed through BLAST analyses against all sequences in GenBank. Sequences were aligned using ClustalW2 and Neighbor joining and Maximum likelihood trees using default parameters were

obtained. Base pair differences were counted with SequencherTM 3.0. Comparisons were made against all existing H3 sequences of *Typhlatya* from the Yucatan accessioned in GenBank, which included two populations of *T. pearsei*: 1) Cenote Nohchen (HE800971) and 2) from an undescribed locality (DQ079702); two populations of *T. mitchelli*: 1) Cenote San Juan (FN995538) and 2) Cenote Hoctun (HE800970); one population of *T. dzilamensis*: Cenote Cervera (HE800972); one population of *Typhlatya* sp.: Cenote Chak Mool (FN995541); one population of *Typhlatya* sp.: Cenote Crustaceo (FN995541). Localities are shown in **Figure 2**. *Typhlatya taina* (HE800980) from Cuba was included as the outgroup since BLAST analyses showed it to be the most similar species to the Yucatan species.
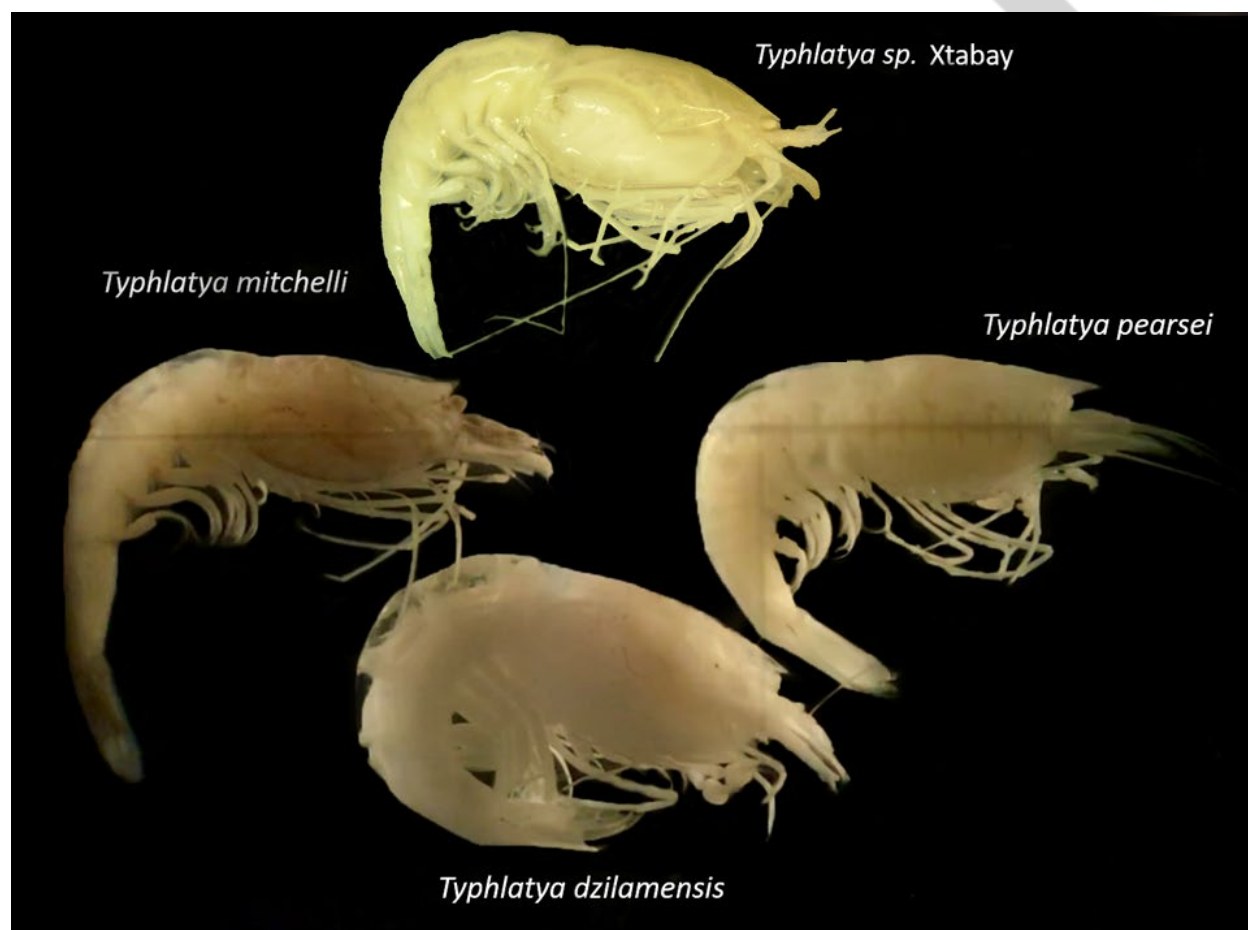

Figure 1. Specimens of previously described species from the Yucatan and Quintana Roo and the newly discovered population from Cenote Xtabay. Photographs by Efraín M. Chávez Solís.
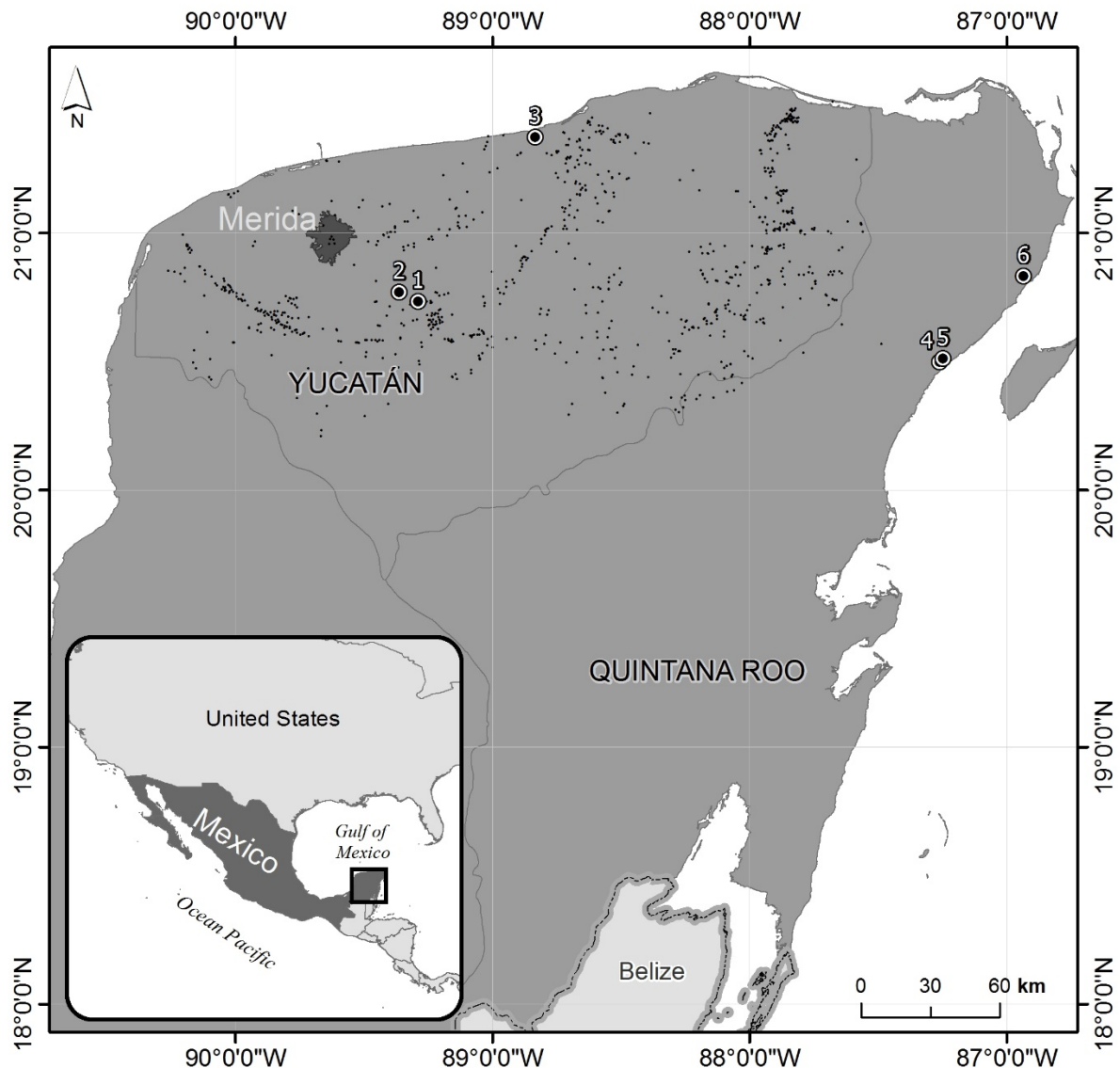
Figure 2. Localities for the populations of *Typhlatya* incorporated in this study: *T. mitchelli*: Cenote San Juan (1) and Cenote Nohchen (2); *T. dzilamensis*: Cenote Cervera (3); *T. pearsei*: Cenote Nohchen (2); *Typhlatya* sp.: Xtabay (4); *Typhlatya* sp.: Cenote Chak Mool (5); *Typhlatya* sp.: Cenote Crustaceo (6). Black dots represent registered cenotes of Yucatan State (SEDUMA).

All H3 sequences for the three Xtabay specimens were identical and 328 bp long. Sequences from Xtabay (population 4 in **Figure 2**) were identical to *Typhlatya* sp. from Cenote Crustaceo (population 6 in **Figure 2**; Genbank # FN995541). Since our sequences of Xtabay specimens were identical to this previously reported sequence, we did not accession our sequences to GenBank. Cenotes Xtabay and Crustaceo are about 50 km apart along the eastern seacoast of Quintana Roo (**Figure 2**). The specimens from both populations were collected in saltwater. Xtabay and Crustaceo sequences were

most similar to *T. dzilamensis* from Cenote Cervera (HE800972), 190 km away, which also inhabits saltwater. They differed by just two out of 328 bp (0.6 %). Differences among all other specimens analyzed and the gene tree are shown in **Table 1** and **Figure 3**, respectively.

Table 1. Uncorrected base-pair differences and sequence divergence among *Typhlatya* populations based on 328-bp of histone 3 (H3).

	T. pearsei	*T. mitchelli*	*T. sp.* ChaK Mool	*T. dzilamensis*	*T. sp.* Crustaceo	*T. sp.* Xtabay
T. pearsei	0 (0.0%)					
T. mitchelli	10-11 (3.0-3.3%)	1 (0.3%)				
T. sp. Chak Mool	8 (2.4%)	0-1 (0.0-0.3%)	–			
T. dzilamensis	21 (6.4%)	17-18 (5.2-5.5%)	15 (4.7%)	–		
T. sp. Crustaceo	20 (6.1%)	16-17 (4.9-5.2%)	14 (4.3%)	2 (0.6%)	–	
T. sp. Xtabay	20 (6.1%)	16-17 (4.90-5.20%)	14 (4.30%)	2 (0.6%)	0 (0.0%)	–
T. taina Cuba	25 (7.1%)	23-24 (7.0-7.3%)	21 (6.4%)	21 (6.4%)	20 (6.1%)	20 (6.1%)

Based on these results, several conclusions can be reached regarding the phylogenetic relationships of the *Typhlatya* populations analyzed:

1) Data strongly support that the unidentified population of *T.* sp. from Cenote Chak Mool sampled by Von Rintelen et al. (2012) is *T. mitchelli*. The specimen from this locality has identical H3 sequences to another population assigned to this species.
2) The populations at cenotes Xtabay and Crustaceo probably belong to the same species as their H3 sequences are identical, they inhabit the same underground saltwater environment, and are in close proximity geographically. The eastern coast of Quintana Roo is known for having extensive underground connectivity, so these two cenotes could be part of the same hydrologic system.
3) In support of previous studies (Botello et al. 2013), there are two major clades of *Typhlatya* in the Yucatán. One clade is comprised of populations that inhabit primarily freshwater (*T. mitchelli* and *T. pearsei*), and the other that inhabits saltwater (*T. dzilamensis*, *T.* sp. Xtabay, and *T.* sp. Crustaceo).
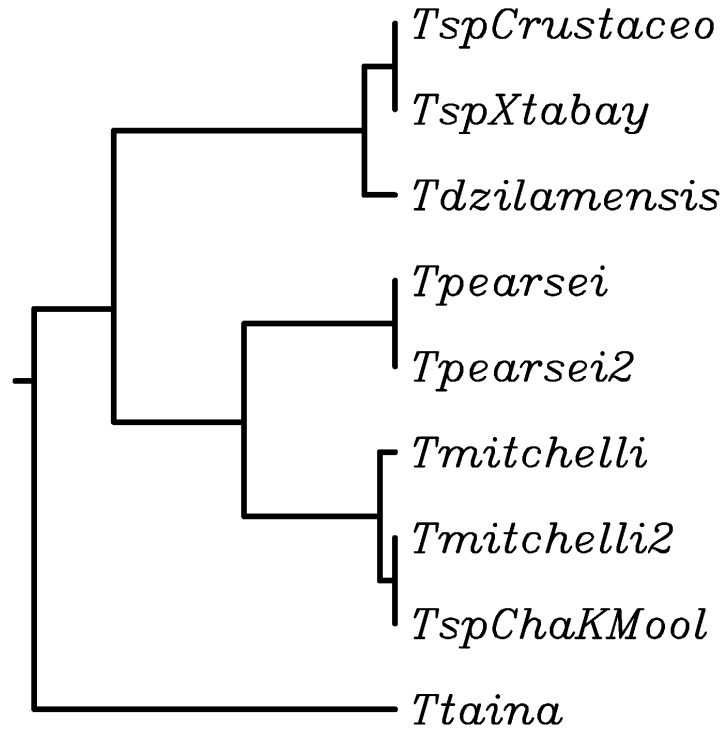
Figure 3. Phylogenetic tree of four described *Typhlatya* species and three unassigned populations. Data is based on histone H3A sequences. Neighbor joining and Maximum likelihood had the same topology.

Based on preliminary morphologic analyses, specimens appeared to be most similar to *T. dzilamensis.* In *T. mitchelli* the rostrum is short, and it does not reach the margin of the eyes. In *T. pearsei* and *T. campecheae* the rostrum is long, and it extends beyond the distal margin of the eyes. In the new population and in *T. dzilamensis* the rostrum is of intermediate length reaching the distal margin of the eyes. Alvares et al. (2005) provided a taxonomic key to the species of the genus *Typhlatya*. The following is a modified key for the species of the Yucatan, in which the populations of Xtabay and Crustaceo fit well within an assignment to *T. dzilamensis.*

1. Rostrum extending beyond distal margins of eyes 2
    Rostrum not reaching or barely reaching distal margins of eyes 3

2. Rostrum reaching first antennular segment...................................*T. campecheae*
    Rostrum reaching second antennular segment .... *T. pearsei*

3. Ratio of carpus/propodus of second pereiopod more than 2.5 ....*T. mitchelli*
    Ratio of carpus/propodus of second pereiopod less than 2.5......... *T. dzilamensis,* Xtabay and Crustaceo populations

Furthermore, Cenote Xtabay and Crustaceo populations share with *T. dzilamensis* its habitat as they all occur in saltwater. The other three species inhabit freshwater. Based on genetics, morphology, and habitat, our results support that the Xtabay and Crustaceo populations belong to *T. dzilamensis*. This constitutes a considerable extension of the range for the species. Previously it had been described as endemic to three nearby cenotes in the central-northern coast of the Yucatan Peninsula, in the state of Yucatan. The new localities extend this range to the eastern coast of Yucatan in the state of Quintana Roo. It may well be that its range extends throughout the coastal region of the Yucatan Peninsula.

Some limitations to our study are that the H3 locus has a slow molecular clock, which could fail to clearly differentiate among sister species. Also, our preliminary morphological analyses were not designed for characters other than those known to hold taxonomic significance in previous studies, so intra- and inter-population variation was not analyzed. Such studies will require further collections to enlarge sample size. Interpretation of our results should be done with caution as we may not be able to resolve between closely related sister species whose differentiation is hidden behind high morphologic similarities and low genetic differentiation. In addition to discovering and sampling additional populations, future research should concentrate on establishing morphological variability within and among populations and to determine genetic variability within and among populations. For example, using multiple mitochondrial markers, Hunter et al. (2008) demonstrated that *Typhlatya* sp. from Cenote San Antonio Chiich is an independent species closely related to *T. mitchelli*.

Hunter et al. (2008) reported that *Typhlatya sp.* from Cenote San Antonio Chiich had very similar sequences and was most closely related to *T. mitchelli*; but was most closely allied with *T. pearsei* based on morphology, with only subtle differences existing between the two. This suggests that the evolutionary environment of the underwater karst of the Yucatan may be leading populations into divergent or convergent pathways that may be difficult to unravel by morphologic comparisons between populations alone. It is encouraged that future research efforts combine data from both genetics and morphology. Such efforts could prevent describing two morphologically variable populations as different species or two distinct species that have converged in their morphological appearance as the same species.

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