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ORIGINAL ARTICLE

Phylogeny and historical biogeography of the cave-adapted shrimp genus *Typhlatya* (Atyidae) in the Caribbean Sea and western Atlantic

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ABSTRACT

Aim To infer phylogenetic relationships among five species of the cave-adapted shrimp genus *Typhlatya* in order to test competing hypotheses of dispersal and colonization of the disjunct cave localities occupied by these five species.

Location *Typhlatya* species are found in caves and anchialine ponds across the northern margin of the Caribbean Sea, along the Mediterranean and Adriatic coasts and on oceanic islands in the Atlantic and eastern Pacific oceans. This study focuses on five species, one from Bermuda, one from the Caicos Islands and three from the Yucatan Peninsula of Mexico.

Methods Partial sequences (*c.* 1400 bp) from the mitochondrial cytochrome *b*, 16S rDNA and COI genes were obtained from representative samples of the five species. Phylogenetic inference was carried out with maximum parsimony and maximum likelihood analyses. Parsimony networks were constructed for the Bermudian species *Typhlatya iliffei* and one Yucatan species *Typhlatya mitchelli*, to determine the degree of connectivity among populations inhabiting different cave systems.

Results All three land masses were recovered as monophyletic. The two insular marine species from Bermuda and the Caicos Islands formed a clade, while the three continental freshwater species from the Yucatan Peninsula formed another. Within both Bermuda and the Yucatan, shared haplotypes were found in different cave systems, suggesting recent or ongoing gene flow among populations in both locales.

Main conclusions The two insular marine *Typhlatya* species originated from an ancestral marine population, possibly already cave-adapted, that is suggested to have colonized the Caicos Islands and subsequently dispersed to Bermuda via the Gulf Stream. Divergence estimates suggest that colonization occurred before the formation of present-day anchialine cave habitat, which did not form on either island until the late Pliocene to early Pleistocene. Divergence estimates also indicate that the Yucatan freshwater species split before the formation of freshwater cave habitat in the Yucatan. These species could have inhabited crevicular marine habitats before the late Pliocene/early Pleistocene in the Yucatan or elsewhere in the Caribbean, and subsequently migrated to freshwater caves once they formed.

Keywords

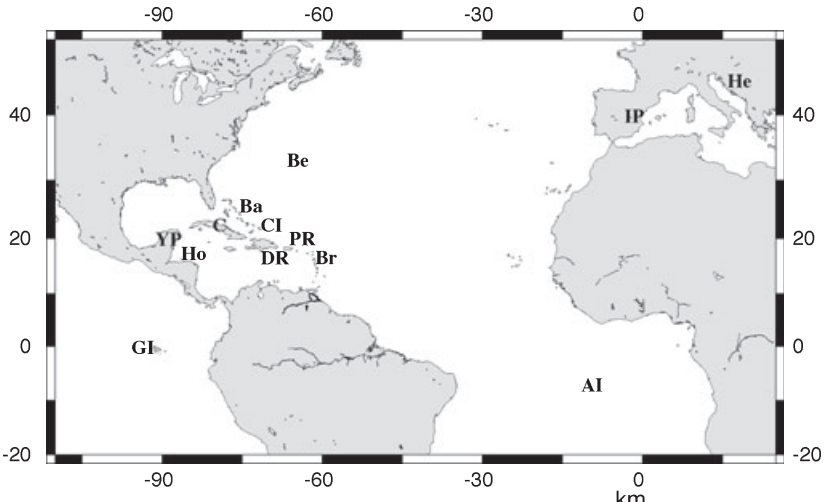
16S rDNA, anchialine, Bermuda, cave-adapted, COI, Crustacea, cytochrome *b*, mtDNA, shrimp biogeography, *Typhlatya*.

INTRODUCTION

Anchialine stygobitic fauna are often characterized by highly disjunct distributions, with members of the same genus or even species inhabiting freshwater to fully marine caves on widely separated islands or continents (Iliffe *et al.*, 1983, 1984). Anchialine refers to coastal caves that have been flooded with seawater but lack a surface connection to the sea (Stock *et al.*, 1986), and stygobites are the aquatic cave-limited forms inhabiting this environment. For example, stygobitic species of the isopod genus *Curassanthura*, amphipod genus *Pseudoniphargus* and copepod genus *Paracyclopia* have amphi-Atlantic distributions (Iliffe, 1993), whereas the ostracod *Iliffeoecia* is reported only from anchialine habitats in Bermuda and the Galapagos Islands (Maddocks, 1991). Anchialine caves in western Australia are inhabited by a number of species congeneric with marine stygobites from the Atlantic, including the thermosbaenacean *Halosbaena*, isopod *Haptolana*, amphipod *Liagoceradocus*, remipede *Lasionectes* and ostracod *Danielopolina* (Humphreys, 2000). Such punctuated distributions have led to competing hypotheses about the age, origin, colonization routes and phylogenetic affinities of morphologically similar taxa inhabiting environmentally comparable cave systems situated thousands of kilometres apart (reviewed by Iliffe, 2000, 2004).

Oceanic dispersal via pelagic larvae has been proposed to explain current distribution patterns of some anchialine fauna (Smith & Williams, 1981; Kano & Kase, 2004). However, many anchialine species are thought to lack a pelagic larval stage (Hart *et al.*, 1985; Manning *et al.*, 1986). Oceanic dispersal of adults has also been postulated (Maciolek, 1983). Another theory suggests that anchialine species formed deep-water continuums between the caves of one island and caves of another island or continent via natural crevices in seafloor bedrock (Hart *et al.*, 1985). This deep-sea hypothesis is supported by a number of anchialine species whose closest morphological relative inhabits the deep sea (Hart *et al.*, 1985). Stock (1986a,b) explained some anchialine distribution patterns with the shallow-water hypothesis, where cave species originated from ancestors inhabiting benthic shallow waters in close proximity to cave habitat. Stock (1977, 1980) also developed the regression model, which states that marine ancestors of stygobiont taxa were stranded by means of tectonic uplift or sea-level drop. Active migration has also been proposed, where expansionistic marine species colonize cave habitat within their range (Danielopol, 1980), while vicariance is thought to have played a role in the disjunct distribution of cave fauna presumed to be Tethyan relicts (Sterrer, 1973; Iliffe *et al.*, 1983).

Similar to other stygobitic taxa, atyid shrimps in the genus *Typhlatya* Creaser, 1936 (Atyidae) have a disjunct distribution (Fig. 1), inhabiting anchialine pools and caves on islands and continental coastal regions across the northern margin of the Caribbean Sea, along the Mediterranean and Adriatic coasts



Code	Locality	Species
AI	Ascension Island	*T. rogersi*
Ba	Bahamas	*T. kakuki*
Br	Barbuda	*T. monae*
Be	Bermuda	*T. iliffei*
CI	Caicos Islands	*T. garciai*
C	Cuba	*T. consobrina, T. elenae, T. garciadebrasi, T. garciai, T. taina*
DR	Dominican Republic	*T. monae*
GI	Galapagos Islands	*T. galapagensis*
He	Hercegovina	*T. pretneri*
Ho	Honduras	*T. utilaensis*
IP	Iberian Peninsula	*T. miravetensis*
PR	Puerto Rico	*T. monae*
YP	Yucatan Peninsula	*T. campecheae, T. dzilamensis, T. mitchelli, T. pearsei*

Figure 1 Map showing the worldwide distribution of all species currently ascribed to the genus *Typhlatya*.

and on oceanic islands in the Atlantic and eastern Pacific oceans. The majority of *Typhlatya* species occur in freshwater, but about one third, including *T. rogersi* from Ascension Island, *T. kakuki* from the Bahamas, *T. iliffei* from Bermuda, *T. garciai* from the Caicos Islands, *T. galapagensis* from the Galapagos Islands and *T. dzilamensis* from the Yucatan Peninsula of Mexico, inhabit brackish to fully marine caves (Sanz & Platvoet, 1995; Alvarez *et al.*, 2005).

Here we focus on phylogenetic relationships of five *Typhlatya* species in an effort to begin to understand their historical biogeography. We also examine local connectivity within two of these species to evaluate population-level processes operating on more recent evolutionary time scales. The five species include two insular species, *T. iliffei* from Bermuda and *T. garciai* from the Caicos Islands, and three continental taxa from the Yucatan Peninsula of Mexico, namely *T. mitchelli*, *T. pearsei* and an undescribed taxon. This latter taxon was identified as an evolutionarily significant unit (ESU) by Webb (2003), based on molecular data. Both insular species inhabit marine caves, whereas the continental taxa inhabit freshwater caves. The objectives of the present study are to elucidate phylogenetic relationships among these five species and test for concordance with geographical distribution. It is hypothesized that the three freshwater taxa from cenotes (water-filled sinkholes) in the Yucatan Peninsula evolved independently of the two insular species that inhabit marine-influenced caves in the western Atlantic Ocean. We sequenced portions of three mitochondrial genes in order to test this hypothesis and ascertain possible patterns of dispersal and subsequent colonization of these geographical localities.

MATERIALS AND METHODS

Samples and DNA sequencing

Typhlatya iliffei specimens were collected while cave diving from two caves on the island of Bermuda during the summer of 2002. Five individuals were collected from the Cliff Pool (CP) section of Green Bay Cave system at 16–17 m depths, and 11 individuals were collected from Tucker's Town Cave (TT) at 9–15 m depths. Additionally, 45 individuals were collected from the 30 m deep saltwater pumping well at the Bermuda Aquarium, Museum and Zoo (BAMZ). *Typhlatya garciai* samples were collected from <2 m depths in the open entrance pool of Old Blue Hills Cave on the island of Providenciales, Caicos Islands in June 2003. *Typhlatya mitchelli*, *T. pearsei* and the ESU *Typhlatya* sp. were collected in July 2000 and 2001 from 10 cenotes in the states of Yucatan and Quintana Roo, Yucatan Peninsula of Mexico. All specimens were preserved in 70% ethanol. DNA sequence data for *T. mitchelli* (AF512032–AF512056; AF513509–AF513538), *T. pearsei* (AY115531–AY115539) and the ESU *Typhlatya* sp. (AY115540–AY115549) were downloaded from GenBank. Four specimens tentatively identified as *Antecaridina lauensis*, collected in an anchialine pond from the Marshall Islands, along with a *Halocaridina rubra* (NC008413) sequence from GenBank, were used as outgroups. Species, collection localities and GenBank accession numbers are summarized in Table 1 and shown for the ingroup in Fig. 2.

Genomic DNA was isolated from 50 mg muscle tissue using Tris–HCl, EDTA and NaCl solution (TENS) and proteinase K. Primers used for both PCR and sequencing were CO9 (5′-TTCGGTCAYCCAGAAGTMTAT-3′) and CO10 (5′-TAAGCGTCTGGGTAGTCTGARTAKCG-3′) for the cytochrome oxidase I gene (COI) (Baldwin *et al.*, 1998), 16SarL (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SbrH (5′-CCGGTCTGAACTCAGATCACGT-3′) for 16S rDNA (16S) (Palumbi *et al.*, 1991) and Cyb1 (5′-ATTTGTCGAGATGTRAAYTAYGG-3′) and Cyb2 (5′-AAATATCATTCNGGYTGRATRTG-3′) for the cytochrome *b* gene (cytb) (Harrison, 2001). PCR cycling parameters for COI and 16S consisted of an initial denaturation step at 94°C for 3 min followed by 35 cycles at 94°C for 45 s, 47°C for 45 s, 72°C for 1 min with a final extension phase at 72°C for 5 min. For the cytb fragment, cycling parameters included an initial denaturation step at 94°C for 2 min followed by 35 cycles at 94°C for 20 s, 50°C for 30 s, 72°C for 30 s with a final extension phase at 72°C for 3 min. Purified sequencing products were run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Three mitochondrial gene fragments were sequenced for each of the five species, with the exception of the ESU *Typhlatya* sp., for which COI sequence data could not be obtained.

Phylogenetic analyses

DNA sequences were edited using BIOEDIT sequence alignment editor ver. 5.0.9 (Hall, 1999) coupled with CHROMAS ver. 1.45 (McCarthy, 1996). Sequences were aligned using CLUSTALX ver. 1.81 (Thompson *et al.*, 1997) with gap-opening penalty = 15 and gap-extension penalty = 30, and adjusted manually where necessary. COI and cytb sequences were translated into amino acids to ensure there were no stop codons to prevent the inclusion of nuclear pseudogenes in analyses. For 16S, alignments required the addition of a small number of gaps, and regions of ambiguous homology were excluded from analyses. 16S, COI and cytb alignments are available in TREEBASE (Accession no. S1873). COLLAPSE ver. 1.2 (Posada, 2004) was used to collapse individual sequences into representative haplotypes for phylogenetic analyses.

Phylogenetic analyses using both maximum parsimony (MP) and maximum likelihood (ML) were carried out in PAUP* version 4.0b5 (Swofford, 2002). A partition-homogeneity test (PHT) (Farris *et al.*, 1995; Huelsenbeck *et al.*, 1996) was implemented in PAUP* to verify that the three mitochondrial gene fragments (COI, cytb and 16S) were congruent with respect to phylogenetic signal. Heuristic search parameters for the PHT were 1000 replicates with 10 random addition sequence replicates. Subsequently, analyses were done on a three-gene concatenated data set (COI + cytb + 16S) and a two-gene concatenated data set (cytb + 16S). The two-gene data set was used to determine the relationship of the ESU

Table 1 Details of specimens, collection localities and sequences used in this study (taxon codes refer to those used in Figs 2–5).

Taxon	Code	Cave locality	GenBank accession no.		
			COI	16S	cytb
Typhlatya iliffei	CP	Cliff Pool, Bermuda	DQ863176–DQ863177	DQ863146–DQ863147	DQ900834-DQ900835 DQ863150
T. iliffei	TT	Tucker's Town Cave, Bermuda	DQ863178–DQ863182	DQ863148–DQ863149	DQ900836-DQ900840 DQ863151–DQ863154
T. iliffei	BW	BAMZ Well, Bermuda	DQ863183–DQ863191	DQ863155–DQ863165	DQ900841–DQ900848
T. garciai	BH	Old Blue Hills Cave, Providenciales, Caicos Is.	DQ863170–DQ863175	DQ863136–DQ863145	DQ900825–DQ900833
T. mitchelli	SJ	Cenote San Juan, Yucatan, Mexico	AF513518–AF513520	AF513533–AF513535	AF512045–AF512051
T. mitchelli	SAC	Cenote San Antonio, Yucatan, Mexico	AF513516–AF513517	AF513531–AF513532	AF512043–AF512044
T. mitchelli	CJ	Cenote Chi Juan, Yucatan, Mexico	AF513513–AF513515	AF513528–AF513530	AF512037–AF512042
T. mitchelli	K	Cenote Kakuel, Yucatan, Mexico	AF513521–AF513523	AF513536–AF513538	AF512052–AF512056
T. mitchelli	CW	Cenote Carwash, Quintana Roo, Mexico	AF513510–AF513512	AF513525–AF513527	AF512033–AF512036
T. mitchelli	N	Cenote Naharon, Quintana Roo, Mexico	AF513509	AF513524	AF512032
T. pearsei	SM	Cenote Santa Maria, Yucatan, Mexico	AY115536	AY115539	AY115533
T. pearsei	27	Cenote 27 Steps, Quintana Roo, Mexico	AY115535	AY115538	AY115532
T. pearsei	AA	Cenote Aayin Aak, Quintana Roo, Mexico	AY115534	AY115537	AY115531
Typhlatya sp.	SAY	Cenote San Antonio Chiich, Yucatan, Mexico	n/a	AY115540–AY115544	AY115545–AY115549
cf. *Antecaridina lauensis*	MI	Anchialine pond, Jaluit Atoll, Marshall Is.	DQ863192–DQ863195	DQ863166–DQ863169	DQ900849–DQ900851
Halocaridina rubra	HI	Hawaii	NC008413	NC008413	NC008413

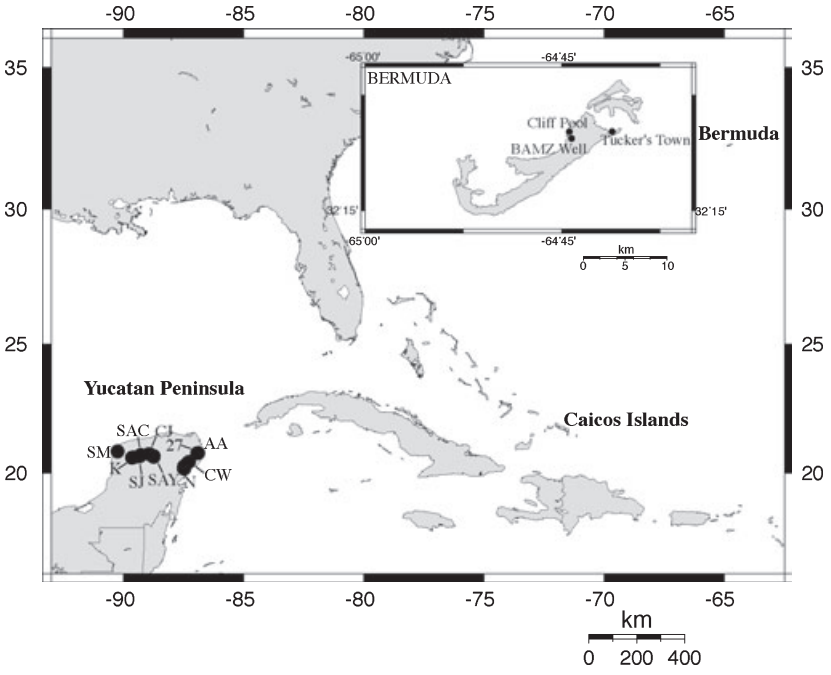


Figure 2 Map showing the three geographical localities (Bermuda, Caicos Islands and the Yucatan Peninsula of Mexico) where *Typhlatya* species were collected for this study.

Typhlatya sp. to the other *Typhlatya* species, as COI data were not available for *Typhlatya* sp.

Maximum parsimony searches were run under two conditions. In the first, transitions and transversions were weighted equally; in the second only transversions were included. Heuristic search parameters for both conditions included tree bisection and reconnection branch-swapping with random addition sequence replicates. Gaps were treated as missing data. For ML, MODELTEST ver. 3.7 (Posada & Crandall, 1998) was used to select the best-fit model of

sequence evolution using the Akaike information criterion. The transversional model with a gamma-shape parameter of 2.1375 and proportion of invariable sites of 0.4517 was selected for the three-gene data set, while the Tamura–Nei model with a gamma-shape parameter of 1.2534 and proportion of invariable sites of 0.3812 was selected for the two-gene data set. Nodal support for MP and ML topologies was estimated using nonparametric bootstrapping (Felsenstein, 1985) with 1000 replicates.

Genetic distances and intraspecific analysis

Genetic distances and standard errors were calculated in MEGA ver. 3.1 (Kumar *et al.*, 2004) on single-gene data sets. The Tamura–Nei model was used on the single-gene data sets, as this was the model available in MEGA determined to be closest to the model chosen by MODELTEST. For 16S and COI, the alpha value of the gamma parameter specified by MODELTEST was implemented in the Tamura–Nei model. Genetic distances were calculated as net average genetic distances, which accounts for within species/clade polymorphism. Divergence rates were taken from prior studies that have calibrated a molecular clock using fossil/geological information for COI and 16S from a variety of decapods. A relatively narrow range of mutation rates has been reported for these mitochondrial genes; it was therefore assumed it was appropriate to extrapolate these rates for *Typhlatya*. For COI, sequence divergences ranging from 1.4% (Knowlton & Weigt, 1998) to 2.3% per million years (Knowlton *et al.*, 1993; Brower, 1994 for *Heliconius*; Daniels *et al.*, 2002) have been reported, and for 16S divergences ranging from 0.65% (Schubart *et al.*, 1998) to 0.9% per million years (Sturmbauer *et al.*, 1996) have been reported. TCS ver. 1.18 (Clement *et al.*, 2000) was used to construct haplotype networks in order to evaluate intraspecific relationships among *T. iliffei* individuals from Bermuda and *T. mitchelli* individuals from the Yucatan Peninsula. A 95% connection limit was used between haplotypes.

RESULTS

In total, the three-gene data set included 1389 bp (12 characters excluded) of which 618 were variable and 554 were parsimony-informative. For the three-gene transversion parsimony, 343 characters were variable and 285 were parsimony-informative. The three-gene data set included 36 ingroup individuals represented by 29 unique haplotypes. For *T. mitchelli* there were nine haplotypes from 15 individuals; for *T. pearsei* there were three haplotypes from three individuals; for *T. garciai* there were two haplotypes from three individuals; and for *T. iliffei* there were 12 haplotypes from 12 individuals. The two-gene data set included 930 bp (19 characters excluded) of which 433 were variable and 391 were parsimony-informative. The two-gene transversion parsimony included 243 variable characters and 199 parsimony-informative characters. For this data set, a total of 45 ingroup individuals were used, from which there were 36 unique haplotypes. For *T. mitchelli* there were seven haplotypes from 15 individuals; for *T. pearsei* three haplotypes from three individuals; for the ESU *Typhlatya* sp. two haplotypes from five individuals; for *T. garciai* seven haplotypes from eight individuals; and for *T. iliffei* there were 14 haplotypes from 14 individuals.

Phylogenetic relationships and genetic distances

The results of the two- and three-gene MP and ML analyses are shown in Figs 3 & 4, respectively. The PHT for the three gene fragments was not significant ($P = 0.864$), suggesting that they do not differ in phylogenetic signal.

All MP and ML topologies supported monophyly of each of the five *Typhlatya* species with 99–100% bootstrap support. A sister relationship for the insular marine species *T. garciai* and *T. iliffei* was supported with moderate bootstrap values (75% and 69%) in the two- and three-gene transversion parsimony analyses, and with slightly higher values (88% and 77%) in the

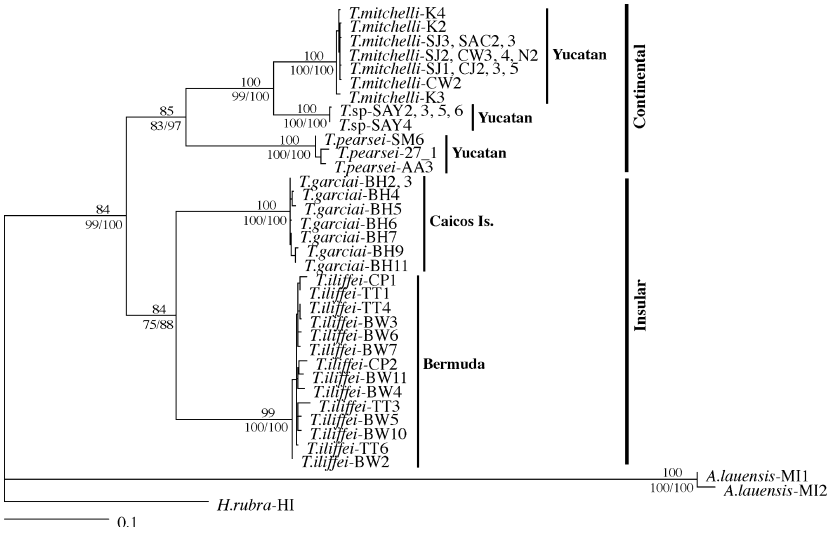


Figure 3 Maximum likelihood (ML) two-gene tree. Cave localities are listed after species name and correspond to codes given in Table 1. Numbers above and below nodes correspond to bootstrap support values from ML and maximum parsimony analyses, respectively. For numbers below nodes, transversion-only bootstrap values are listed first, followed by equally weighted parsimony bootstrap values.

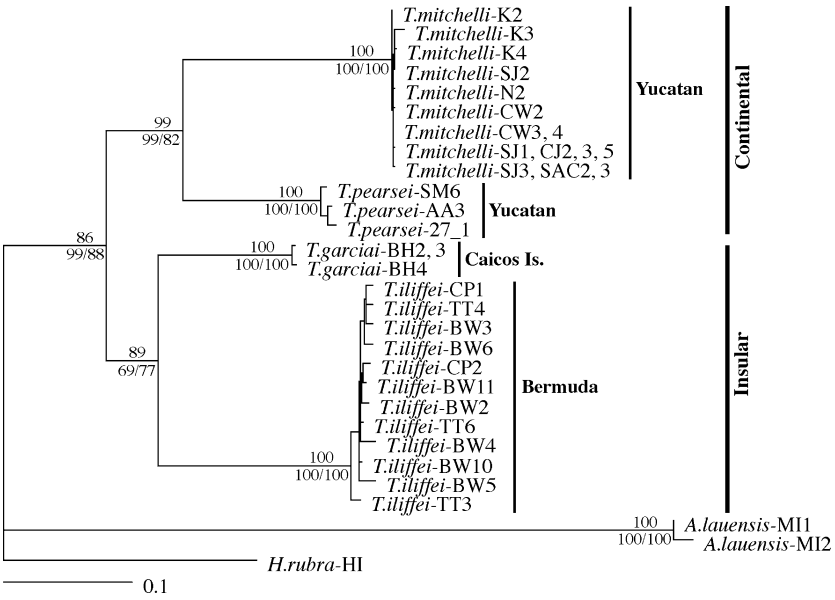


Figure 4 Maximum likelihood (ML) three-gene tree. Cave localities are listed after species name and correspond to codes given in Table 1. Numbers above and below nodes correspond to bootstrap support values from ML and maximum parsimony analyses, respectively. For numbers below nodes, transversion-only parsimony bootstrap values are listed first, followed by equally weighted parsimony bootstrap values.

two- and three-gene equally weighted parsimony analyses. Both ML analyses recovered this sister relationship as well, with 84% and 89% bootstrap support for the two- and three-gene analyses, respectively. All MP and ML topologies strongly supported a monophyletic Yucatan clade with bootstrap values >80%. Of the three Yucatan taxa, *T. mitchelli* and the ESU *Typhlatya* sp. were strongly supported (bootstrap proportions ≥99%) as sister taxa in all topologies in which the ESU taxon was included (all two-gene topologies).

Genetic distances between the four species described were high, ranging from 17% to 33% (Table 2). Intraspecific distances were comparatively very low, ranging from 0% to 3%, within the range of values reported for other atyids (Hurwood & Hughes, 2001; Chenoweth & Hughes, 2003; Hurwood *et al.*, 2003; Santos, 2006). Using COI and 16S divergence rates stated previously with COI and 16S divergences reported in Table 2, the split between the freshwater continental and marine insular clade was inferred to occur between 23 and 32 Ma with 16S, and between 12 and 20 Ma with COI. For the Yucatan taxa, 16S genetic distances indicated an early divergence of *T. pearsei* from the *T. mitchelli*/*Typhlatya* sp. clade (22–31 Ma), whereas COI data suggested a much more recent split (9–16 Ma) for *T. pearsei* and *T. mitchelli*. According to the 16S data, *T. mitchelli* and the ESU *Typhlatya* sp. diverged around 7–9 Ma, while 16S and COI analyses indicate that the two insular species, *T. garciai* and *T. iliffei*, split from one another *c.* 11–28 Ma.

Table 2 16S and COI percentage sequence divergences between *Typhlatya* species and clades, with SE in parentheses.

	16S	COI
T. mitchelli vs. ESU	5.9 (1.4)	n/a
T. mitchelli/ESU vs. *T. pearsei*	20.1 (3.8)	21.8 (3.0)
T. garciai vs. *T. iliffei*	18.5 (3.5)	26.0 (3.7)
Island vs. continental	20.9 (3.7)	28.3 (3.8)

Sequence divergences were calculated as net average genetic distances under the Tamura–Nei model with the gamma parameter.

Typhlatya iliffei and *T. mitchelli* intraspecific relationships

The *T. iliffei* cytb haplotype network analysis (Fig. 5) resulted in a single network with an ancestral haplotype (identified with a rectangle) shared by three individuals, two from Tucker's Town Cave (TT) and one from BAMZ Well (BW). Another cytb haplotype was shared by one individual from TT and one individual from BW. The remaining 10 haplotypes in the network represented single individuals. Haplotype networks constructed with 16S and COI sequences for *T. iliffei* (data not shown) were largely congruent with that of cytb. The 16S haplotype network had an ancestral haplotype shared by seven individuals from all three Bermudian collection localities. Two individuals with this haplotype were from Cliff Pool (CP), two were from TT and three were from BW. The other 12 16S haplotypes sampled for *T. iliffei* were one to a maximum of three steps away from the ancestral haplotype. The COI sequences resulted in four parsimony networks. However, three networks contained only one individual, each from BW, while the remaining 13 individuals comprised a single network. Additionally, one COI haplotype was shared by an individual from TT and from BW.

The *T. mitchelli* cytb haplotype network analysis (Fig. 5) similarly resulted in a single network. The presumed ancestral haplotype was shared by nine individuals from four cenotes, two (San Juan (SJ) and San Antonio (SAC), located centrally in the northern region of the peninsula and two (Carwash (CW) and Naharon (N)) located on the eastern margin (see Fig. 2 for exact locations). The numerically dominant cytb haplotype was connected by one step to the ancestral haplotype and shared by 10 individuals from two cenotes (SJ and Chi Juan (CJ)) in northern central Yucatan. The six remaining haplotypes were

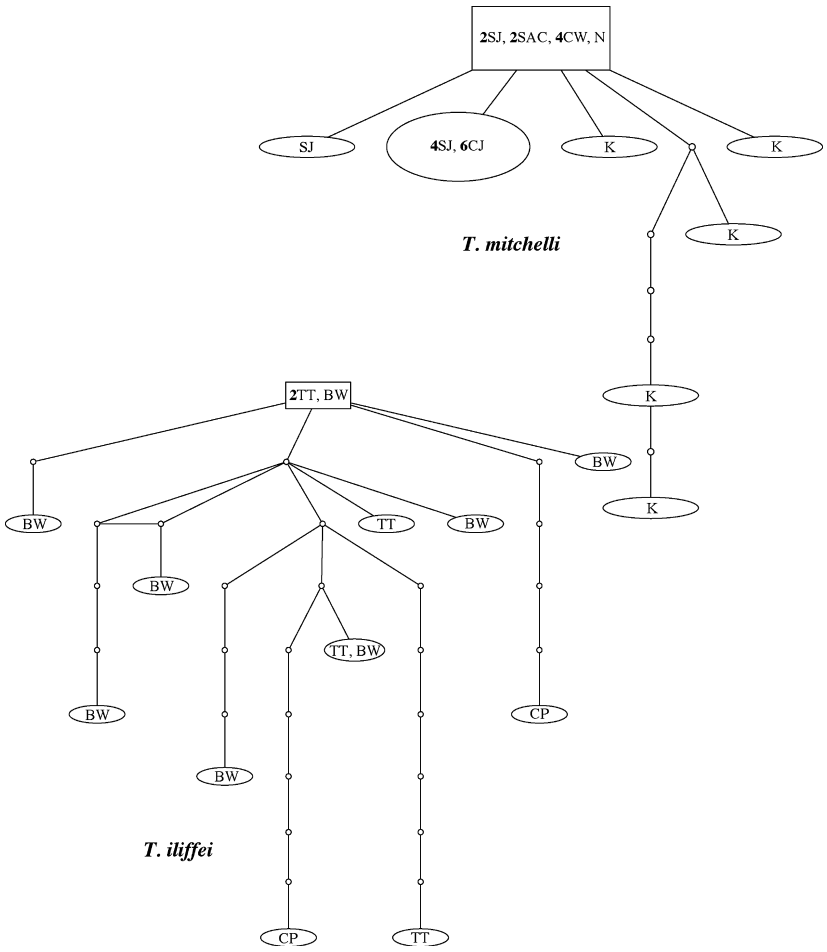


Figure 5 Cytochrome *b* parsimony networks of *Typhlatya iliffei* individuals collected from Bermuda and *Typhlatya mitchelli* individuals collected from the Yucatan. Letters inside ovals/rectangles indicate collection locality for each haplotype and correspond to Table 1. The frequency of each haplotype is indicated numerically inside ovals/rectangles unless the frequency = 1. Rectangles indicate the presumed ancestral haplotype.

found from single individuals. The 16S network (data not shown) was comprised of only three haplotypes, all of which were connected by a single step. The ancestral haplotype was shared by 11 individuals from five cenotes (Kakuel (K), CJ, SJ, CW and N) and the two connecting haplotypes were comprised of three individuals from two cenotes (SJ and SAC) and one individual (CW). For COI (data not shown), a single network of four haplotypes was recovered. The ancestral haplotype was shared by 11 individuals from five cenotes (K, CJ, SJ, CW and SAC), with two haplotypes separated by one step. One of these haplotypes was shared by two individuals from two cenotes (K and SJ) and the other was a single individual from Naharon. A single haplotype from Kakuel was separated by five steps from the K/SJ haplotype.

DISCUSSION

Phylogenetic relationships

The two- and three-gene MP and ML topologies support a sister relationship for *T. garciai* and *T. iliffei*, both found in brackish to fully marine anchialine habitats on islands in the Atlantic Ocean/Caribbean Sea. *Typhlatya mitchelli* and the ESU *Typhlatya* sp. were also recovered as sister taxa that inhabit freshwater caves in the northern region of the Yucatan Peninsula. The phylogenetic position of the third Yucatan species, *T. pearsei*, was sister to *T. mitchelli* in the three-gene topologies and sister to *T. mitchelli*/*Typhlatya* sp. in the two-gene topologies.

The ESU population (*Typhlatya* sp.) was designated a provisional species (Webb, 2003) due to high interspecific divergences with the four other *Typhlatya* species relative to its intraspecific divergence. A value of 10 times the average intraspecies divergence in the COI gene has been proposed for designating molecular operational taxonomic units (Hebert *et al.*, 2004). Although COI data were not available for the ESU, the average intraspecific divergence for 16S was 0.2%, whereas the average distance between *Typhlatya* sp. and *T. mitchelli*, its closest congener, was 5.9%, well above 10 times the intraspecific distance. High interspecific divergence (13.6%) was also found between *Typhlatya* sp. and *T. mitchelli* for cytochrome *b*. Species status of the ESU is further supported by reciprocal monophyly of this taxon in all topologies, supported by 100% bootstrap values. It is evident that the single ESU population sampled represents an undescribed species of *Typhlatya* that is most closely related to *T. mitchelli*, a more widely distributed species from the Yucatan Peninsula. Interestingly, examination of *Typhlatya* sp. revealed that this species is most closely allied with *T. pearsei* based on morphology, with only subtle differences existing between the two. For example, both species

have the unique character of reduced exopods on the fifth pereiopod, with *Typhlatya* sp. showing more dramatic reduction in this trait. Other morphological differences between the two species include rostrum length, antennal scale length, size and shape of the distolateral uropod spine and number of spines on the distal margin of the telson (F. Alvarez, personal communication).

Biogeographical scenarios

Yucatan Peninsula of Mexico

Divergence dates calculated for *Typhlatya* species indicate that the ancestral split between insular species (*T. garciai* and *T. iliffei*) and continental species (*T. mitchelli*, *T. pearsei* and *Typhlatya* sp.) occurred during the middle Oligocene–middle Miocene. It is unknown whether this ancestor was epigean (living above ground) or had already evolved into a stygobitic form. Although caves presently inhabited by these species in all three localities did not begin forming until late Pliocene–early Pleistocene (Wilkens, 1979; Iliffe *et al.*, 1983), almost certainly caves and/or crevicular habitats that could house anchialine taxa have existed since the early stages in the formation of these land masses. The presence of primitive, relict taxa including remipedes (thought to be most primitive of living crustaceans), *Erebonectes* (one of the most primitive calanoid copepods) and *Antriscopia* (similar in many ways to the theoretical ancestral copepod) reflect the great age of anchialine habitat (Iliffe, 2004). Therefore it is possible that the ancestor was already stygobitic. Furthermore, considering that all atyids are thought to have a marine origin (Creaser, 1936) and that the insular clade is marine, it is likely that this ancestor was also marine. The most parsimonious explanation for the occurrence of the three species in the Yucatan freshwater cave system is that they evolved from a single marine ancestor that colonized the Yucatan. Following colonization, speciation was probably promoted by geographical isolation within the extensive subterranean system. It has been suggested that genetic isolation often occurs upon colonization of cave habitat, as some caves are separated from one another by substantial distance and 'deep-sea arms' that are difficult to traverse by shallow, warm water-adapted crustaceans (Sanz & Platvoet, 1995).

The ESU *Typhlatya* sp. has been collected from only one cenote (San Antonio Chiich cenote, SAY) where it does not co-occur with any other *Typhlatya* species. This indicates that *Typhlatya* sp. became geographically isolated post-ancestral colonization of the Yucatan and after divergence of *T. pearsei* from the *T. mitchelli*/*Typhlatya* sp. lineage. This undescribed species may be endemic to SAY. Conversely, although not sampled in this study, some populations of *T. mitchelli* and *T. pearsei* are known to be sympatric (Wilkens, 1979). Present-day sympatry of these two species is probably attributed to secondary contact between species that evolved in previously isolated subterranean systems, as suggested by Cobolli Sbordoni *et al.* (1990).

The Yucatan Peninsula began developing *c.* 200 Ma and consists of at least a 2400 m thickness of limestone of shallow-water origin (Smart *et al.*, 2006). Formation of presently known caves was initiated as early as the late Pliocene (Wilkens, 1979). Sea-level fluctuations during glacial–interglacial periods alternately exposed and submerged caves until they eventually became water-filled in the late Pleistocene as sea levels rose due to melting of Northern hemisphere ice caps (Reddell, 1977). Alvarez *et al.* (2005) estimated the age of *T. mitchelli* and *T. pearsei* to be *c.* 5–6 Myr based on the time of emergence of the Yucatan Peninsula. Divergences from this study indicate that *T. pearsei* and the *T. mitchelli*/*Typhlatya* sp. lineage diverged soon after the continental/insular split, although a wide range of divergence times were calculated for the *T. pearsei* split. 16S data indicated a 7–9 Myr divergence date for *T. mitchelli* and *Typhlatya* sp.; however, COI data were not available for *Typhlatya*. sp. Given that COI suggested more recent dates for all divergences considered, it is possible that *T. mitchelli* and *Typhlatya* sp. split more recently than the latest date suggested by 16S (7 Ma). The major implication of these divergence times is that the Yucatan taxa split before the formation of present-day freshwater cave habitat, which formed no earlier than the late Pliocene (1–2 Ma). If divergence times are correct, then the Yucatan freshwater cave system must have been colonized independently by each species. Multiple freshwater invasions have been shown in other crustaceans (Lee, 1999). The three Yucatan species could have inhabited crevicular marine habitats before the late Pliocene/early Pleistocene in the Yucatan or elsewhere in the Caribbean, and subsequently migrated to freshwater caves once they formed. Conversely, mutation rates used to calculate divergence times may not be appropriate for stygobites or *Typhlatya*. For example, if a higher rate of mitochondrial sequence evolution is occurring in *Typhlatya*, then divergence times are overestimates and these three species may not have split until after formation of freshwater cave habitat.

Bermuda and the Caicos Islands

Divergence times for the insular species indicate that dispersal rather than vicariance contributed to the distribution of these two taxa, especially as the mid-Atlantic island of Bermuda has never been part of or nearer to any continental land mass than it is at present. Bermuda was formed at least 30–40 Ma by volcanic activity in the Atlantic oceanic crust, and has been emergent since the Miocene–Pliocene boundary, *c.* 6 Ma (Rowe, 1998). Bermuda's caves are situated in Pleistocene-age limestone that caps the summit of the volcanic seamount. The Bahamian archipelago, to which the Caicos Islands belong, began developing *c.* 200 Ma, concurrent with the opening of the Atlantic Ocean (Carew & Mylroie, 1995), and similarly to Bermuda and the Yucatan, its presently known caves are of Pleistocene origin (Gregor, 1981).

Bermuda has been colonized largely by taxa originating from the Caribbean and Gulf of Mexico carried to the island by

prevailing north-easterly winds and the Gulf Stream (Monniot & Monniot, 1983; Williams & Williams, 1999). Based on mean speed estimates derived from Weyl (1970), Stock (1986c) estimated that it would take *c.* 15 days to travel from the Bahamas to Bermuda via the Gulf Stream. Therefore, the marine ancestor of *T. garciai* and *T. iliffei* could have colonized the Caicos Islands and been carried by the Gulf Stream to Bermuda and independently adapted to anchialine habitat in both localities. This scenario would support the 'shallow-water' hypothesis (Stock, 1986a,b). Alternatively, the ancestor could have invaded cave habitat in the Caicos Islands, and adults or larvae of this newly stygobitic form could have been dispersed to Bermuda, where it colonized similar habitat. This explanation is more parsimonious as only a single adaptation to anchialine habitat is required. It has been hypothesized that one of the major colonization pathways for certain atyid cave shrimps is dispersal, mainly of larvae, through the marine environment (Stock, 1986c). Larval forms are not known for any *Typhlatya* species, but the Hawaiian anchialine atyid *H. rubra* has long-lived pelagic larvae (Couret & Wong, 1978), indicating that this scenario might be possible for *Typhlatya*. It should be emphasized here that while *T. garciai* occurs in both fresh and brackish cave waters, only individuals found in the brackish cave waters of the Caicos Islands were analysed in this study, and the analysis of Cuban freshwater populations could provide further insight into the evolution and dispersal of this species.

Typhlatya iliffei and *T. mitchelli* intraspecific relationships

Few studies have investigated the connectivity of anchialine populations occurring in different caves on the same land mass (Webb, 2003; Kano & Kase, 2004; Santos, 2006). In this study, genetic distances within *T. iliffei* were found to be a maximum of 3.1% for COI, 1.7% for cytb and 0.8% for 16S. While COI intraspecific distances were relatively high, they do correspond with other reported intraspecific distances for atyids, including *Caridina* (Hurwood & Hughes, 2001), *Halocaridina* (Santos, 2006) and *Paratya* (Hurwood *et al.*, 2003). For COI, intrageneric comparisons were no less than 21%, reflecting the high evolutionary rate of this mitochondrial region (Mueller, 2006) and explaining why intraspecific distances were as high as 3.1% in some cases. This also explains why the COI haplotype analysis did not cluster all *T. iliffei* individuals into a single network. However, the COI haplotype network representing the majority of haplotypes included individuals from all three collection localities, and 16S and cytb haplotype analyses grouped all *T. iliffei* individuals into a single network. These analyses, coupled with the fact that identical haplotypes were collected from all three localities, strongly suggests that at least these three caves on the island of Bermuda are connected via the hypogeal (underground) water system. This level of connection has also been reported for the anchialine atyid *H. rubra* (Santos, 2006), where population differentiation occurred in populations separated by at least 30 km. The connectivity of the three cave systems in Bermuda is across <30 km. BW is separated from CP by only 380 m, and from TT by 4700 m, and CP and TT are separated from each other by 4900 m. Genetic exchange across greater geographic distances has also been reported for cave fauna. For example, dispersal by ocean currents has been shown to facilitate gene flow across distances as great as 200 km in a stygobiont gastropod species (Kano & Kase, 2004). This scale of genetic connectivity was found in this study for *T. mitchelli* throughout the northern Yucatan Peninsula. Haplotype analyses resulted in networks where most haplotypes were connected by single steps, indicative of low population differentiation. Further, identical haplotypes were collected in multiple cenotes, some separated by as much as 235 km. Shared haplotypes across such a large geographic distance suggest hypogeal connectivity throughout the Yucatan Peninsula, at least for this species. Greater sampling of *T. pearsei* throughout the Yucatan would allow population connectivity to be evaluated in this species as well.

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