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Cryptic diversity in a widespread live-bearing fish (Poeciliidae: *Belonesox*)

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Belonesox belizanus Kner (Teleostei: Poeciliidae) is a wide-spread livebearing species that occurs on the Atlantic Slope of Central America from southern Mexico to northern Costa Rica. Previous work has noted morphological variation within the species, and recognized two subspecies: *Belonesox belizanus belizanus* and *Belonesox belizanus maxillosus*. We used 1122 bp of cytochrome *b* and 617 bp of S7-1 DNA to conduct a phylogeographical study of *Belonesox*, aiming to examine the genetic distinctiveness of these taxa and other populations of *Belonesox* throughout the range. Bayesian phylogenetic and haplotype analyses indicated that *B. b. maxillosus* is not distinctive from other northern populations of *Belonesox*. However, a distinct phylogeographical break is evident near the Rio Grande in southern Belize. One clade comprises the putative *B. b. maxillosus* and all populations sampled north of the Rio Grande. The other clade comprises the Rio Grande and all populations south thereof. Fossil-calibrated divergence time estimates suggest that isolation of the northern and southern lineages of *Belonesox* occurred approximately 14.1 Mya. The phylogeographical structure recovered in the present study is interesting, considering that relatively few studies have examined molecular variation across this portion of Middle America in a time-calibrated framework. Furthermore, the present study suggests that more work is needed to adequately understand the factors that have shaped diversity of this region. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2013, **109**, 848–860.

ADDITIONAL KEYWORDS: Belize – biogeography – cyt *b* – DNA – phylogeography – poeciliid – subspecies.

INTRODUCTION

The viviparous Family Poeciliidae [Rosen & Bailey, 1963 = Poeciliinae (Parenti, 1981)], Order Cyprinodontiformes, includes more than 20 genera and more than 200 species (Lucinda, 2003; Lucinda & Reis, 2005). The family includes some of the most common aquarium species, such as swordtails (*Xiphophorus* spp.), mollies (*Poecilia* spp.), and the guppy, (*Poecilia reticulata*), as well as lesser known species, including a subterranean sulfur spring variety of *Poecilia mexicana* (Tobler *et al*., 2008; Plath & Tobler, 2010) and *Tomeurus gracilis*, a species that lays internally fertilized eggs (Parenti & Rauchenberger, 1989; Lucinda & Reis, 2005; but see also Ghedotti, 2000). Poeciliids occur in both fresh and brackish waters of North and CentralAmerica, and the West Indies, Africa, and Madagascar (Rosen & Bailey, 1963). Poeciliids are ubiquitous throughout this region, being found in lakes, rivers, streams, estuaries, and subterranean habitats.

The pike killifish, *Belonesox belizanus*, is a predatory poeciliid species that was described by Kner (1860). The exact location of the type locality is unknown because the original description only lists 'Belize' as the collection locality for the holotype. *Belonesox* is a monotypic genus, distinctive in morphology (Fig. 1), and is the largest poeciliid, reaching a maximum length of 200 mm (Lee *et al*., 1980; Page & Burr, 1991, 2011). This species is widespread and occurs from the Trans-Mexican Volcanic Belt at the Río de La Antigua system in Veracruz, Mexico, south along the Gulf Coastal Plain, and into northern Costa Rica (Belshe, 1961; Rosen & Bailey, 1963; Miley, 1978; Reis, Kullander & Ferrais, 2003; Miller, Minckley & Norris, 2005; Smith & Bermingham, 2005). The

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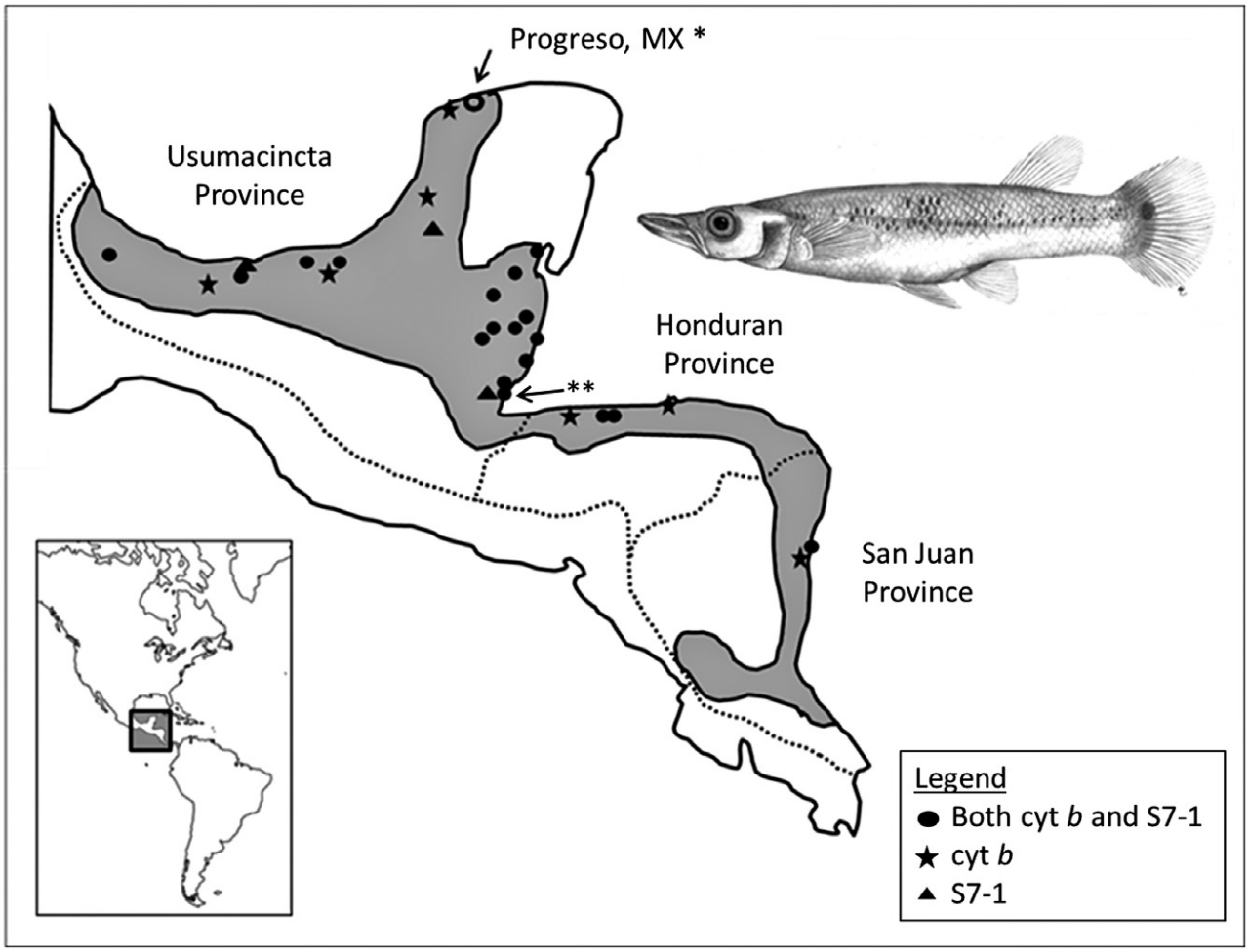

Figure 1. Illustration of *Belonesox belizanus* with its shaded range (Miller, Minckley & Norris, 2005). Invasive populations in southern Florida are not shown. Dotted lines outline the three relevant ichthyological provinces proposed by Miller (1966), Bussing (1976), and Hulsey and López-Fernández (2011). **Belonesox belizanus maxillosus* population. **Rio Grande in Belize. Locations for samples are given in Table 1. MX, Mexico.

range spans three Central American ichthyological provinces: the Usumacincta, Honduran, and San Juan (Fig. 1) (Miller, 1966; Bussing, 1976; Hrbek, Seckinger & Meyer, 2007). Within the Usumacincta province, two major (Usumacincta-Grijalva and Papaloapan-Coatzacoalcos) and two lesser divisions (Yucatan Peninsula and Polochic-Lake Izabal) exist (Miller, 1966; Collette, 1974). The Yucatan Peninsula is unique in that it lacks an abundant amount of surface water and here *Belonesox* can only be found in lagoons and cenotes along the Gulf of Mexico coast. According to Miller (1966) and Myers (1966), *Belonesox* may have originated in the Usumacincta Province, although the division is not specified. Furthermore, Miller (1966) stated that poeciliids likely evolved in Central America because most of the tribes are found there. However, recent molecular work (Hrbek *et al*., 2007) estimated divergence times and phylogenetic relationships with Poeciliidae, and the results supported a South American origin for the family with several dispersal events into Central America.

In 1936, Carl Hubbs described a new subspecies, *Belonesox belizanus maxillosus*, from the Yucatan Peninsula, at the town of Progreso, Mexico. The description was based on < 50 specimens and the specific range of the new form was not formally identified by Hubbs (1936). Hubbs' description states that the subspecies from Progreso is distinct from all other Middle American populations of *Belonesox* as a result of its more 'robust' and 'very large, broad, and heavy jaws'. He did not identify any gonopodial characters, commonly used in poeciliid taxonomy, to support recognition of this new taxon. Phylogenetic studies exploring the monophyly of *Belonesox* will help determine the evolutionary history of this species, thus

allowing the elevation of *B. b. maxillosus* to species level or synonymizing it with *B. belizanus*.

Furthermore, *Belonesox belizanus* was introduced into southern Florida in 1957, likely from source populations in the Yucatan. According to Belshe (unpubl. thesis, 1961), the source stock for the invasive Florida population is from populations of *B. b. maxillosus* near Progreso. As an invasive species in Florida, a large amount of work has been carried out assessing its ability to disperse (Kerfoot, Lorenz & Turingan, 2011), reproduce (Turner & Snelson, 1984), consume prey (Anderson, 1980), and tolerate various environmental conditions (Kerfoot, 2012). Currently, no genetic work has been carried out aiming to substantiate the claim that the invasive Florida populations are from the Yucatan Peninsula.

The objectives of the present study are two-fold. First, to analyze genetic variation within the widespread, native range of *Belonesox belizanus*. The results of this portion of the study will provide information on the genetic distinctiveness of *B. b. maxillosus* from the Yucatan Peninsula and identify the source lineage for the invasive Florida population. Second, a divergence time analysis will be utilized to investigate the timing of divergence among the populations of *Belonesox*, as well as to date the divergence between *Belonesox* and its sister lineage, *Gambusia*. These data will be added to the growing body of knowledge regarding Central America, its history, and biota.

MATERIAL AND METHODS

Taxon sampling

A total of 36 specimens, from 18 localities were collected in Florida, Mexico, Belize, Honduras, and Nicaragua (Fig. 1, Table 1). Fin clips were preserved in 95% ethanol, and voucher specimens were fixed in 10% formalin and archived in natural history museums. Location information for each individual specimen used in the analysis is provided in Table 1. Museum abbreviations are used *sensu* Sabaj Pérez (2012).

Gene sequencing

Whole genomic DNA was isolated using the DNeasy tissue kit (Qiagen Inc.) and then used as a template for polymerase chain reaction (PCR). Each 25 μL cytochome *b* (cyt *b*) PCR reaction consisted of: 0.75 μL of MgCl; 2.5 μL of 10 × buffer; 0.5 μL of dNTPs; 1.0 μL of each 10 μM primer; 0.25 μL of Taq; 1–3 μL of DNA template; and 16–18 of μL water. The primers used for cyt *b* amplification were H15915 and L14724 from Hrbek *et al*. (2007). The protocol for the cyt *b* gene was: initial denaturation at 95 °C for 30 s; 25 cycles of 95 °C for 60 s, 48 °C for 30 s, 72 °C for 105 s; and a final extension at 72 °C for 240 s.

Each 25 μL S7-1 PCR reaction consisted of: 0.75 μL of MgCl; 2.5 μL of 10 × buffer; 0.5 μL of dNTPs; 1.0 μL of each 5 μM primer; 0.25 μL of Taq; 0.5–3 μL of DNA template; and 15.5–18 μL of water. The primers used for the S7-1 amplification were S7F (RP1) and S7R (PEX2R) (Chow & Hazama, 1998). The protocol for the S7-1 gene was: initial denaturation at 94 °C for 240 s; 30 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s; and a final extension at 72 °C for 240 s.

PCR products were electrophoresed on a 1% agarose gel and compared to the standard to assess the quality and presence of fragments. Amplified products were purified for sequencing using Exo-Sap™ (Qiagen Inc.). DNA sequencing was performed by Yale University DNA Analysis Facility. Sequences were edited and aligned by eye using SEQUENCHER, version 4.9 (Gene Codes Corporation).

Phylogenetic analysis

Phylogenetic analyses were performed using Bayesian inference (BI) methods for each dataset independently. A phylogram was reconstructed for cyt *b* and S7-1. Each dataset included the following poeciliids as outgroups: *Poecilia latipinna*, *Heterandria formosa*, and *Gambusia affinis*.

MODELTEST, version 3.7 (Posada & Crandall, 1998) was performed on each gene dataset. For the cyt *b* sequences, the data file was partitioned by codon with a model of evolution chosen for each codon position using the Akaike information criterion (Posada & Buckley, 2004). The nuclear marker, S7-1, was not partitioned and the model of evolution was chosen using the same approach. Posterior probabilities were estimated using Markov chain Monte Carlo methods (Geyer, 1991) in MrBayes (Huelsenbeck & Ronquist, 2001). Each BI was run using MrBayes for 10 000 000 generations with trees sampled every 100 generations. To assess burn-in, likelihood scores versus generation were plotted in Excel (Microsoft Corp.) to determine the point in which stabilization occurred. The first 10% of the BI run was discarded as burn-in. The remaining trees were utilized to determine posterior probabilities and four separate runs for each gene were compared to confirm convergence of estimated topologies.

Haplotype analysis

TCS, version 1.21 (Clement, Posada & Crandall, 2000) was used to examine relationships among all of the cyt *b* haplotypes and among all of the S7-1 haplotypes. The resultant cyt *b* and S7-1 haplotypenetworks were created using default settings and a 95% connection limit.

Table 1. Specimens used for phylogenetic analyses

Code	Museum number	Latitude	Longitude	Drainage	Genbank accession number	
					Cyt *b*	S7-1
1	SLU 1956	18.532	–88.325	Rio Hondo, MX	JX556381	JX679645
2	SLU 2107	19.299	–90.471	Rio Champoton, MX	JX556382	–
3	SLU 2773	18.142	–92.933	Grijalva Usumacincta, MX	JX556383	JX679650
4	NA	25.394	–80.501	Florida, USA	JX556384	JX682939
5	SLU 1983	21.158	–90.027	Sisal, Yucatan; MX	JX556385	–
6	SLU 1839	18.166	–88.683	Rio Hondo, MX	JX556386	JX679646
7	SLU 2778	18.480	–88.480	Rio Hondo, MX	JX556387	–
8	OSUS*-MT11-03	18.134	–93.285	Grijalva Usumacincta, MX	JX556388	–
9	OSUS*-MT11-05	18.116	–92.972	Grijalva Usumacincta, MX	JX556389	JX682941
10	SLU 3395	17.977	–94.114	Rio Tonala, MX	JX556375	JX679659
11	SLU 1607	18.519	–96.429	Rio Papaloapan, MX	JX556390	JX679643
12	SLU 1608	18.519	–96.429	Rio Papaloapan, MX	JX556391	JX679644
13	SLU 2769	17.833	–94.560	Rio Coatzacoalcos, MX	JX556376	–
14	SLU 3377	17.187	–88.999	Belize River, BZ	JX556392	JX679657
15	SLU 3217	17.301	–88.554	Sibun River, BZ	JX556393	JX682942
16	SLU 3154	16.919	–88.344	Silk Grass Creek, BZ	JX556394	JX679652
17	SLU3166	17.404	–88.458	Sibun River, BZ	JX556395	JX679655
18	SLU 3155	16.919	–88.344	Silk Grass Creek, BZ	JX556396	JX679653
19	SLU 3242	16.635	–88.498	Jenkins Creek, BZ	JX556397	JX679654
20	SLU 3201	16.360	–88.793	Golden Stream, BZ	JX556398	JX679642
21	SLU 3289	17.087	–89.127	Belize River, BZ	JX556399	JX679658
22	LSUMZ 2609	13.421	–83.355	Rio Prinzapolka, Nic	JX556400	JX679649
23	LSUMZ 2639	13.421	–83.599	Rio Prinzapolka, Nic	JX556401	–
24	LSUMZ 2638	13.421	–83.599	Rio Prinzapolka, Nic	JX556402	–
25	LSUMZ 3487	15.969	–85.861	Laguna Guaimoreto, HD	JX556403	–
26	LSUMZ 3701	15.717	–87.600	Ulua River, HD	JX556404	–
27	LSUMZ 1051	15.766	–86.999	Rio Papaloteca, HD	JX556405	JX679648
28	LSUMZ 1242	15.667	–87.083	Rio Papaloteca, HD	JX556406	JX679660
29	LSUMZ 1052	15.766	–86.999	Rio Papaloteca, HD	JX556407	–
30	LSUMZ 3488	15.969	–85.861	Laguna Guaimoreto, HD	JX556408	–
31	SLU 3297	16.219	–88.928	Rio Grande, BZ	JX556409	JX679656
32	SLU 3096	16.230	–88.944	Rio Grande, BZ	JX556410	JX679661
33	SLU 2770	18.080	–94.080	Rio Coatzacoalcos, MX	JX556377	JX682940
34	SLU 2775	18.633	–90.283	Grijalva Usumacincta, MX	JX556378	JX679651
35	LSUMZ 1059	15.766	–86.999	Rio Papaloteca, HD	JX556379	–
36	SLU 3038	16.230	–88.944	Rio Grande, BZ	JX556380	JX679641

Code column correlates to the code in each [cytochrome *b* (cyt *b*) and S7-1] phylogenetic tree. Also given are museum and tissue numbers, latitude, longitude (both in decimal degrees), drainage, and GenBank accession number for each gene. *Denotes tissue in Tobler Laboratory collection. BZ, Belize; HD, Honduras; MX, Mexico; NA, not available.

Divergence time estimation

To estimate divergence times within and among populations of *Belonesox* and other Cyprinodontiformes, the cyt *b* dataset was analyzed using BEAST, version 1.7.1 (Drummond *et al*., 2012) with a relaxed lognormal molecular clock (Drummond *et al*., 2006) and a Hasegawa, Kishino and Yano substitution model. The fossil record for poeciliids and New World Cyprinodontiformes is poor. The oldest fossil is a Poeciliidae indet from the Maiz Gordo Formation, Argentina (Cione, 1986) and is of Paleocene origin (58–55 Mya). We took a conservative approach and used this fossil to calibrate the Cyprinodontiformes node, inclusive of Anablepidae, Fundulidae, Poeciliidae, and Profundulidae. Representative Genebank sequences from these families were included in the analysis, including two outgroup taxa (*Basilichthys semotilus* JQ282024 and *Oryzias latipes* AB084750) and several other basal cyprinodontiform taxa, including

Profundulus oaxacae JQ254933, *Profundulus kreiseri* JQ254935, *Fundulus notatus* GQ119744, *Fundulas olivaceus* GQ119744, *Anabelps anableps* EF017508, *Jenynsia lineata* EF0107509, *Priapella compressa* EF017554, *Xiphophorus helleri* EF017548, *Carlhubbsia stuarti* EF017532, *Poecilia latipunctata* EF017539, and *Gambusia affinis* EF017514. Because paleontological data were used, the calibration point was given a lognormal prior (Drummond *et al*., 2006; Ho, 2007) with a minimum age of 55 Mya. The upper bound, based on the maximum age of the Atherinomorpha was set to 75 Mya (mean = 6.4, logstdev = 1.0, offset = 55.0) *sensu* Santini *et al*. (2009). Two independent analyses were performed with 50 000 000 generations, each with trees sampled every 1000 generations. Both sets of log files were analyzed in TRACER, version 1.5 (Rambaut & Drummond, 2007) to verify stationarity and to assess that effective sample size (ESS) values were > 200. After removing 10% of burn-in each tree file, both runs were compared to assure that posterior probability values of all nodes were within 5% from the other run. The tree file from the second run was then annotated in TREEANNOTATOR (Drummond & Rambaut, 2007) where a 95% maximum clade credibility tree was generated and then further analyzed in FIGTREE, version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

RESULTS

Molecular variation

Including the outgroups, the aligned mitochondrial gene sequences, cyt *b*, were truncated to 1122 bp long and had 384 variable characters of which 257 of those were parsimony-informative. All aligned nuclear gene sequences, S7-1, were 617 bp in length and had 27 parsimony informative characters out of 116 variable characters.

Phylogenetic analysis

BI of a 50% majority rule consensus tree for the cyt *b* dataset (1122) resulted in two major clades for *Belonesox* (Fig. 2). The mean uncorrected divergence between clades was 7.5%. One clade includes all populations in drainages north of the Rio Grande in Belize. The other clade comprises all populations within or south of the Rio Grande. The Rio Grande occurs in the southern portion of Toledo District, Belize and is just north of the Chortis-Mayan block boundary at the Motagua-Polochic Fault Zone. The Sisal population, approximately 20 miles west of Progreso and a part of the subspecies *B. b. maxillosus*, falls out in the northern clade and is 0.175%–2.1% divergent from other northern clade populations. The Sisal population is 0.26% divergent from the Florida population.

BI analysis of the S7-1 dataset showed a similar north versus south bifurcation (Fig. 3) with > 95% support; however relationships among the geographical areas were not resolved. The 'North of Belize' clade has a fixed difference at bp 316 and the 'South of Belize' clade has a fixed difference at bp 409. Variation within the S7-1 dataset was low, with 0–3-bp differences within the entire *Belonesox* clade, which equated to a 0.5% sequence divergence between the North and South clades. Within the southern clade, there is another allele present that has one additional difference at bp 369 in the Rio Papaloteca, Honduras. All specimens from within Belize were recovered as an unresolved polytomy. *Belonesox b. maxillosus* from near Progresso could not be amplified for this gene. The Florida population has the same allele as the north clade and thus 0.0% divergence.

Haplotype analysis

The 95% connection limited haplotype networks for cyt *b* reconstructed for the present study support the phylogenetic analysis. Six unconnected networks were resolved for cyt *b*, each correlating to a clade in the phylogenetic analysis (Table 2; Figs 2, 4). S7-1 was not as variable, with only four unique haplotypes (Fig. 4). The source haplotype for S7-1, which was also the most common ($N = 18$), included every Belizean individual and two northern individuals. The second most common haplotype ($N = 10$) included the rest of the northern population.

Divergence time estimation

High effective sample size values (> 200 ESS) were recovered for all parameters in BEAST. Divergence time estimation of the cyt *b* dataset is shown in a 95% maximum clade credibility tree (Fig. 5). The resultant tree recovered two main clades corresponding to the mitochondrial (mt)DNA BI analysis. Divergence time estimates indicate divergence of the family Poeciliidae from other cyprinodontiforms in the Paleocene approximately 59.7 Mya (Fig. 5). *Gambusia* and *Belonesox* then diverged from other poeciliids approximately 44.1 Mya (Node 1, 32.3–53.7 Mya; Fig. 5) and from each other 33.5 Mya (Node 2, 25.2–41.9 Mya; Fig. 5) near the Eocene-Oligocene boundary. Lastly, divergence between the northern and southern clades of *Belonesox* occurred in the mid-Miocene, approximately 14.1 Mya (Node 3, 10.1–18.9 Mya; Fig. 5).

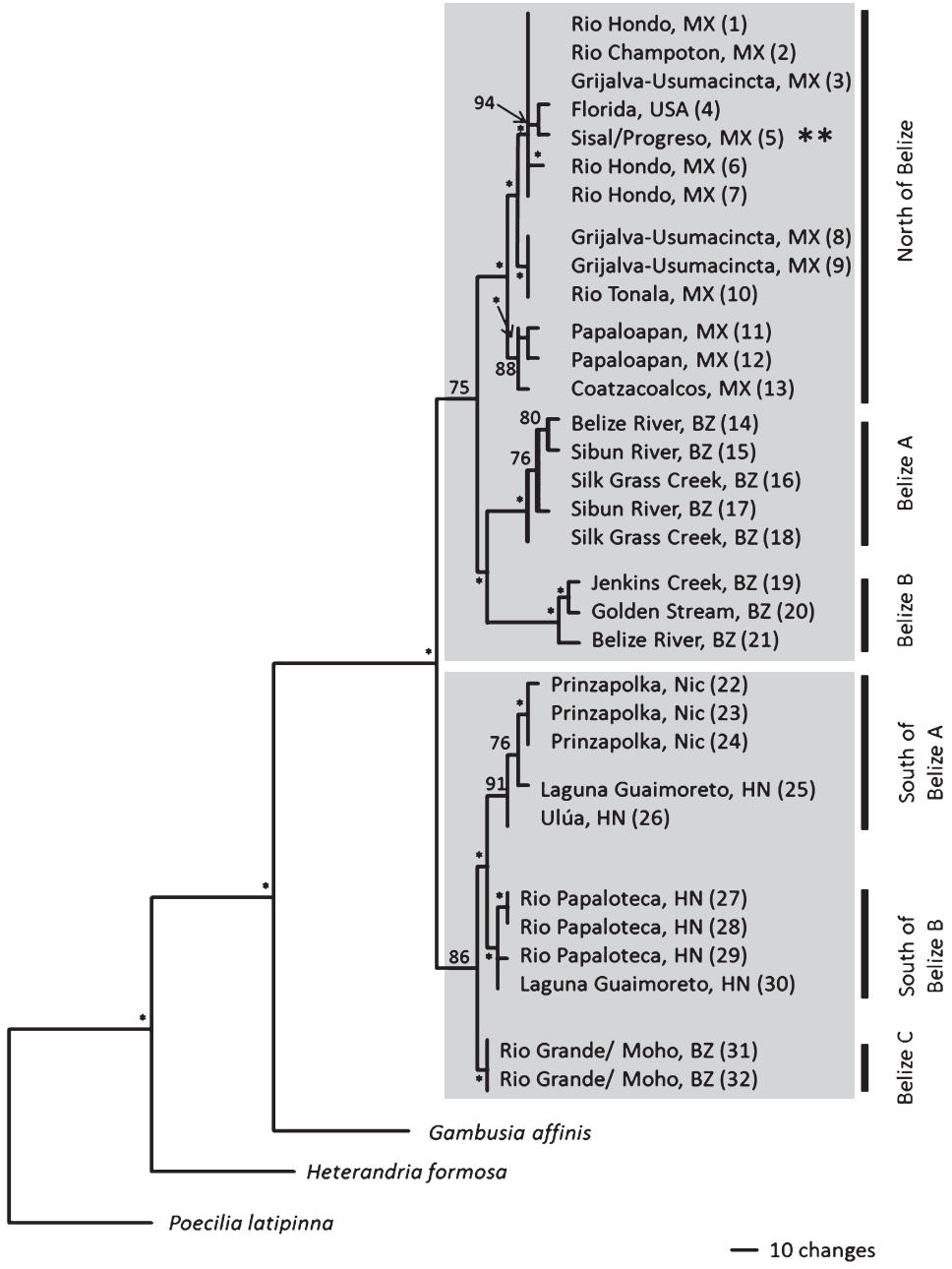

Figure 2. Phylogram of 50% majority rule consensus tree from a partitioned mixed model Bayesian analysis of cytochrome *b* (cyt *b*) sequences (1122 bp). Two major clades were resolved with 7.5% sequence divergence. Tips are given as drainages correlating to the collection location, with the numbers in parentheses corresponding to Table 1. All nodes marked with a single asterisk (*) have posterior probability values ≥ 95. ***Belonesox belizanus maxillosus* population. Designations to the right of the tree correlate to the resolved haplotype networks for cyt *b* (Fig. 4). BZ, Belize; HD, Honduras; MX, Mexico; Nic, Nicaragua.

DISCUSSION

Molecular distinctiveness

The use of molecular data to address biogeographical questions is both character-rich and objective (Perdices *et al.*, 2002) and also adds the ability to estimate divergence times. The successful inference of different levels of a phylogeny via the use of genes that evolve at different rates is a particularly utilitarian aspect of molecular data analysis. mtDNA

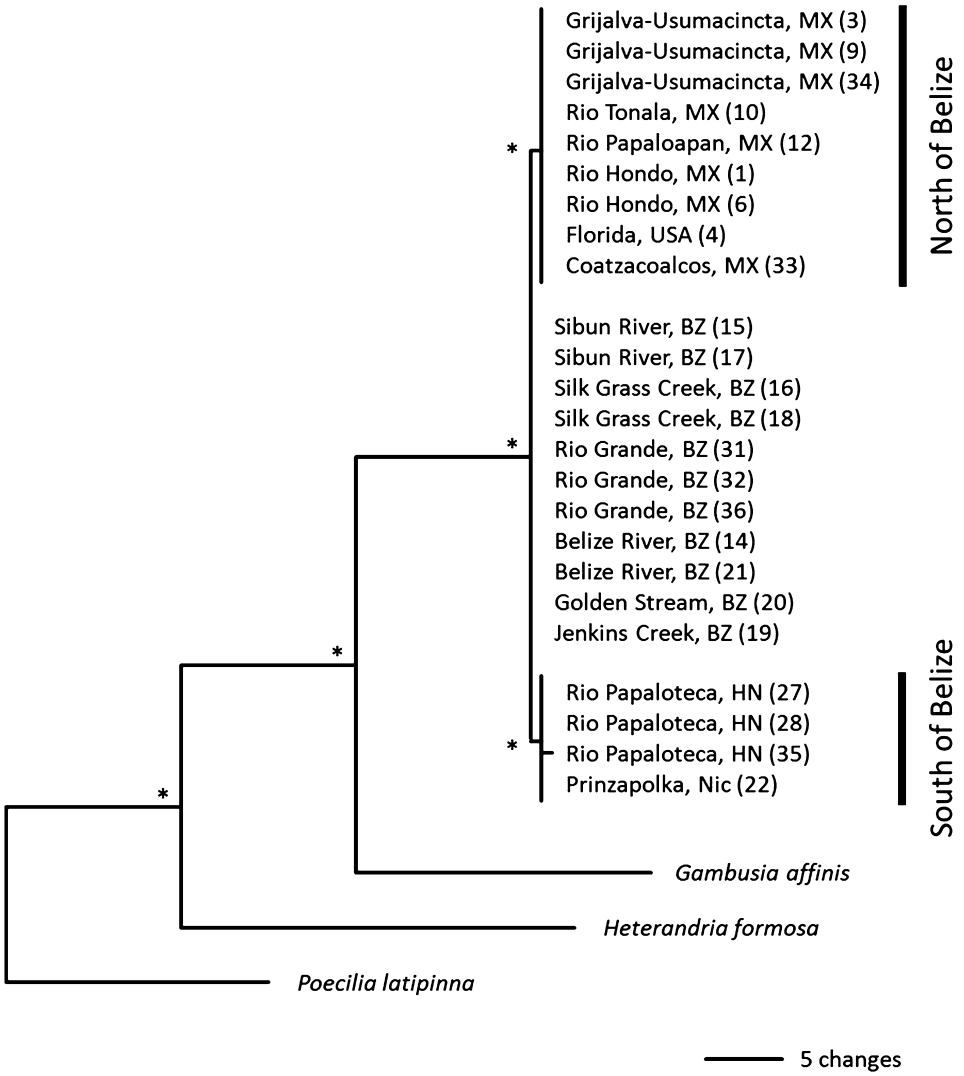

Figure 3. Phylogram of 50% majority rule consensus tree from a partitioned mixed model Bayesian analysis of S7-1 sequences (617 bp). Tips are given as drainages correlating to the collection location, with the numbers in parentheses corresponding to Table 1 and clades correlating to the north versus south divide are depicted with bars. All nodes marked with a single asterisk (*) have posterior probability values ≥ 95. BZ, Belize; HD, Honduras; MX, Mexico; Nic, Nicaragua.

is useful for reconstructing shallow phylogenetic relatedness because it is clonally inherited via the matrilineal line, it is haploid and nonrecombining, and it evolves quickly (Kocher & Stepien, 1997). It is also the favored in intraspecific phylogeographical studies (Avise, Arnold & Ball, 1987; Avise, 1989) if the divergence times correlate to biogeographical events of interest. The use of mtDNA has been criticized because it suggests misleading patterns of variation as a result of hybridization and introgression (Piller, Bart & Hurley, 2008; Keck & Near, 2009), as well as incongruence with nuclear genes as a result of its separate evolutionary history (Bossu & Near, 2009) and incomplete lineage sorting (Maddison, 1997). Specifically, phylogenies that are inconsistent with those derived from nuclear gene sequences in the context of species relationships among closely-related taxa are sometimes produced (Shaw, 2002; Ballard & Whitlock, 2004). Alternatively, nuclear DNA can be used to potentially resolve the deeper levels of the phylogeny and corroborate (or falsify) phylogenetic history of the more rapidly evolving mtDNA (Avise *et al*., 1987; Avise, 1989). By using both mtDNA and nuclear (n)DNA in our intraspecific phylogenetic analysis, a more robust and comprehensive investigation of evolutionary history of *Belonesox* is made (Hrbek *et al*., 2007; Meredith *et al*., 2010).

Table 2. Populations and (codes from Table 1) and their corresponding haplotypes depicted in Figure 4

Gene	Haplotype	Corresponding populations
Cytochrome *b*	1	1
	2	6
	3	2, 34
	4	3
	5	7
	6	5
	7	8, 10, 33
	8	9
	9	12
	10	11
	11	13
	12	16
	13	17
	14	18
	15	15
	16	14
	17	20
	18	19
	19	21
	20	32, 36
	21	31
	22	24
	23	23
	24	22
	25	25
	26	26
	27	30
	28	29, 35
	29	27
	30	28
S7-1	1	14–21, 31–32, 36
	2	1, 3, 6, 8, 10–12, 33–34
	3	22, 28, 35
	4	27

One of the original research questions was to use molecules to explore differences among populations of *Belonesox* and to determine whether *B. b. maxillosus* should be elevated to species or synonymized with *B. belizanus*. The findings of the present study reject the evolutionary distinctiveness of the purported subspecies, *B. b. maxillosus*. Cytochrome *b* shows a mean variation of 2.1% within the northern clade, inclusive of the *B. b. maxillosus* populations from the Yucatan. Specimens from the Yucatan group with other populations from the Rios Papaloapan, Coatzacoalcos, and Hondo basins of Mexico and are concordant with the east–west biogeography found in Mexico by Escalante *et al*. (2007) for many taxonomic groups. Furthermore, the present study corroborates that the source lineage for the invasive south Florida population of *Belonesox* is from the Yucatan Peninsula and is sister to the Sisal/Progreso (*B. b. maxillosus*) specimen with 0.26% divergence between them (cyt *b*; Fig. 2).

The mean intracladal cyt *b* divergence for the southern clade is 1.16%, including the Rio Grande and Moho River systems in Belize, as well as the Ulua River, Laguna Guaimoreto, and Rio Papaloteca in Honduras. This also includes the Prinzapolka River in north-eastern Nicaragua, which is the most divergent population in this clade, with 2.1% variability in cyt *b*. Interestingly, 1.75% variation was found between two specimens collected in Laguna Guaimoreto (Table 1; specimens 25 and 30). However, Laguna Guaimoreto is adjacent to the Caribbean coast in the Colon Department of Honduras and its close proximity to brackish lagoons makes dispersal a possibility. Specimen 30 may have come from another population, such as Rio Papaloteca, and was captured and catalogued from Laguna Guaimoreto. The most notable aspect of the southern clade is the inclusion of two specimens from southern Belize in the Rio Grande drainage. These two specimens are 23.3 km south-west of the Golden Stream population, which is a part of the northern clade.

The nuclear gene phylogeny shows very little variation (0.5%) across the range for the genus *Belonesox*. This low level of variation was somewhat unexpected given that cyt *b* was 7.5% divergent between similar clades; however, other studies incorporating a nuclear marker have recovered similar lower levels of variation relative to mtDNA markers (Hare, 2001; Lee & Edwards, 2008; Lee & Johnson, 2009). The topology of the nuclear gene tree is not as well resolved as the cyt *b* tree, although phylogeographical similarities exist, including a distinct northern and southern clade, with fixed base pair differences at bp 316 and bp 409, respectively. We were unable to amplify the S7-1 gene for purported *B. b. maxillosus* but, based on *cyt b*, it is likely this supspecies is within the northern clade. All Belizean populations were unresolved for S7-1 (Fig. 3). The lack of resolution in the S7-1 tree may be a result of fixation of the nuclear genome and the slow rate of change of nDNA compared to mtDNA. Comparatively, our mtDNA tree has captured intraspecific genetic variation and gene flow.

Central American biogeography

Belonesox belizanus is found in a geologically complex region (Fig. 1) with major fault lines, such as the Motagua-Polochic Fault Zone (Marshall, 2007), and vicariance has likely played a significant role in the biogeographical distribution of freshwater fishes in this region (Perdices *et al*., 2002; Hrbek *et al*., 2007;

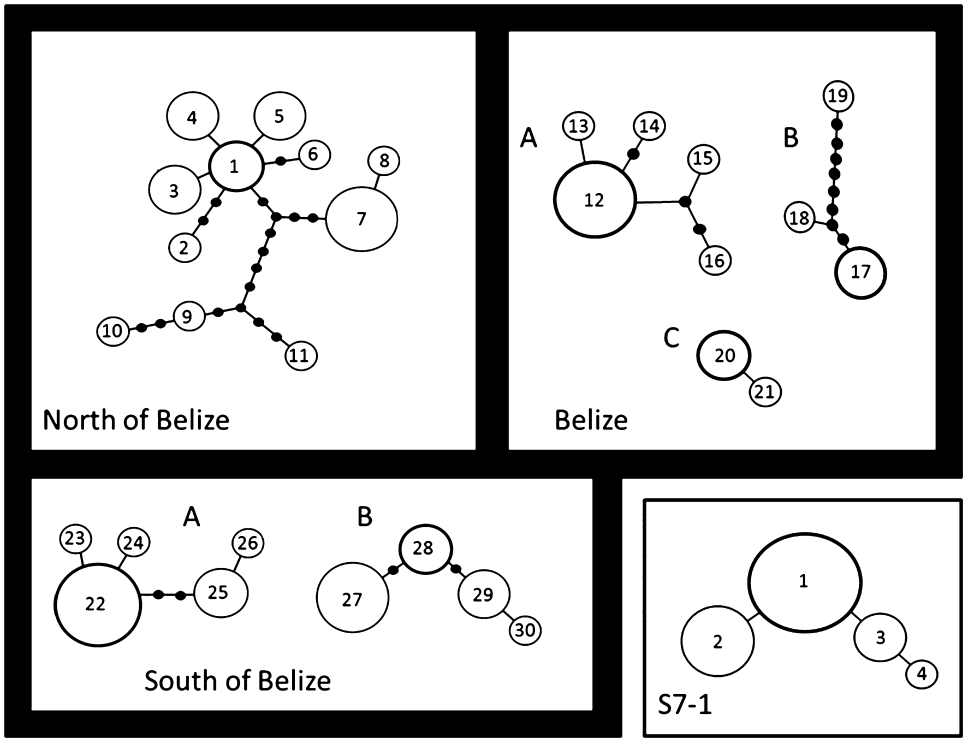

Figure 4. Haplotype networks for cytochrome *b* (cyt *b*) and S7-1 genes with black and white backgrounds, respectively. All cyt *b* haplotype networks are unconnected. Dots represent one base pair change between haplotypes with a 95% connection limit. Numbers correlate to haplotypes in Table 2.

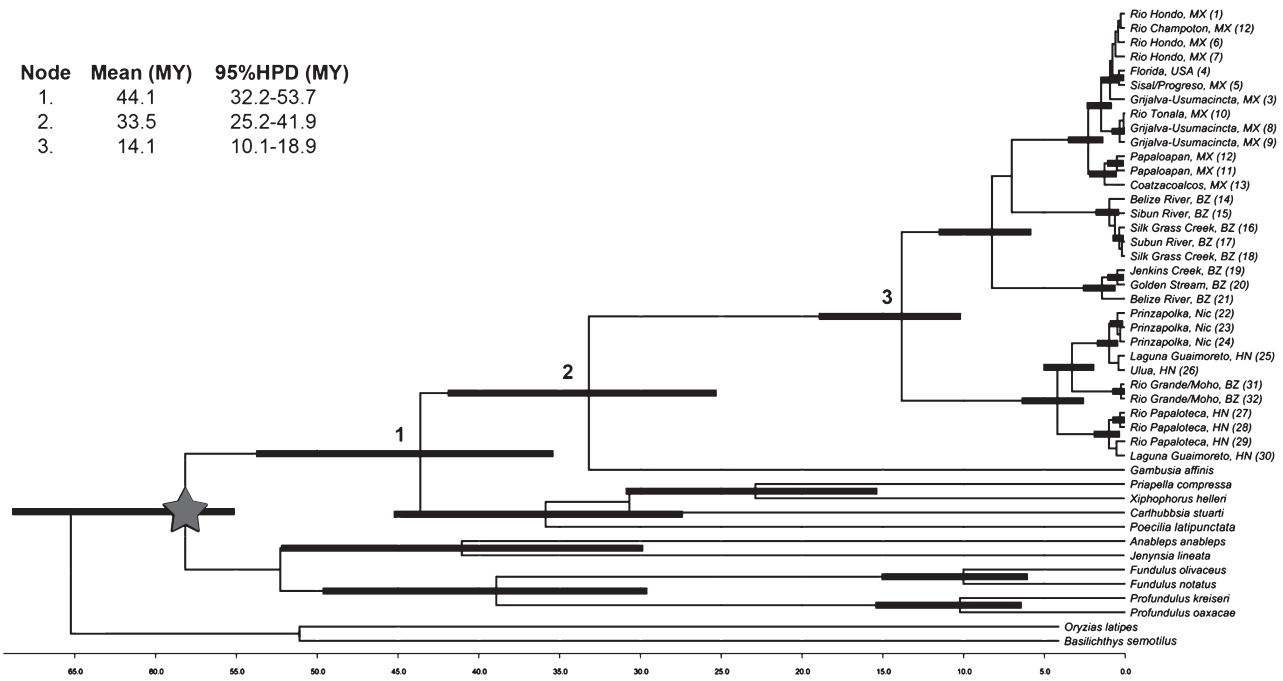

Figure 5. Bayesian divergence times of *Belonesox belizanus* estimated using cytochrome *b* (cyt *b*) and BEAST. Node bars represent 95% posterior age intervals (millions of years). HPD, highest posterior density. Numbers in parentheses correspond to populations in Table 1. Stared node is placement of the fossil calibration and the divergence of poeciliids from other cyprinodontifoms. BZ, Belize; HD, Honduras; MX, Mexico; Nic, Nicaragua.

Hulsey & López-Fernández, 2011; McMahan *et al*., 2013). The uplift of both the Isthmus of Panama in the mid-Pliocene (approximately 3 Mya; Marshall *et al*., 1979) and the Yucatan Peninsula during the Pleistocene allowed Neotropical fishes to expand their ranges into new territory. The Great American Biotic Interchange between North and South America across Central America has occurred in the last 2–7 Myr (Marshall *et al*., 1979; Bermingham, 2002; Tilman, 2011), and has included multiple cycles of colonization and diversification of fishes.

In particular, the timing of diversification of many aquatic groups, specifically with regard to the uplift of the Panamanian Isthmus and other major geological events in the region, has received the most attention (Bermingham & Martin, 1998; Bermingham, 2002; Mateos, Sanjur & Vrijenhoek, 2002; Perdices *et al*., 2002; Smith & Bermingham, 2005; Reeves & Bermingham, 2006; Hulsey & López-Fernández, 2011). Strict molecular clock estimates suggest other salt-tolerant freshwater fishes such as cichlids (Martin & Bermingham, 1998) and *Rivulus* (Rivulidae) (Murphy, Thomerson & Collier, 1999) colonized Mesoamerica from South America between 18–15 Mya (Martin & Bermingham, 1998). This is 10 Myr earlier than primary freshwater fishes (Martin & Bermingham, 1998) and much earlier than the final closure of the Panamanian Isthmus, approximately 3 Mya (Marshall *et al*., 1979; Coates & Obanda, 1996). This was the primary route for dispersal for source populations of freshwater fishes (Myers, 1966; Bussing, 1985; Perdices *et al*., 2002; Hulsey & López-Fernández, 2011).

Our divergence time analysis suggests that poeciliids diversified from other cyprinodontiform families approximately 59.7 Mya [95% highest posterior density (HPD): 55.1–68.8 Mya; Fig. 5, fossil calibrated node]. Hrbek *et al*. (2007), using a semi-parametric penalized likelihood approach to estimate divergence times, noted that the main radiation of poeciliids was approximately 43.9 Mya. Although we do not have the same level of taxon sampling as the study by Hrbek *et al*. (2007), the results of the present study are similar (Node 1, mean = 44.1, 95% HPD: 32.2–53.7 Mya; Fig. 5). These data also show that many poeciliid species were within their current ranges long before the closure of the Panamanian Isthmus, approximately 3 Mya (Marshall *et al*., 1979; Coates & Obanda, 1996). The divergence of *Belonesox* and *Gambusia* from other poeciliids occurred approximately 44.1 Mya (95% HPD: 32.2–53.7 Mya), which is similar to the diversification age (33.9 Mya) of Middle and North American poeciliids as suggested by Hrbek *et al*. (2007). Furthermore, approximately 14.1 Mya (95% HPD: 10.1–18.9 Mya), *Belonesox* split into northern and southern clades near the Rio Grande in Toledo, Belize (Fig. 6, no. 7) where they has been little to no recent gene flow.

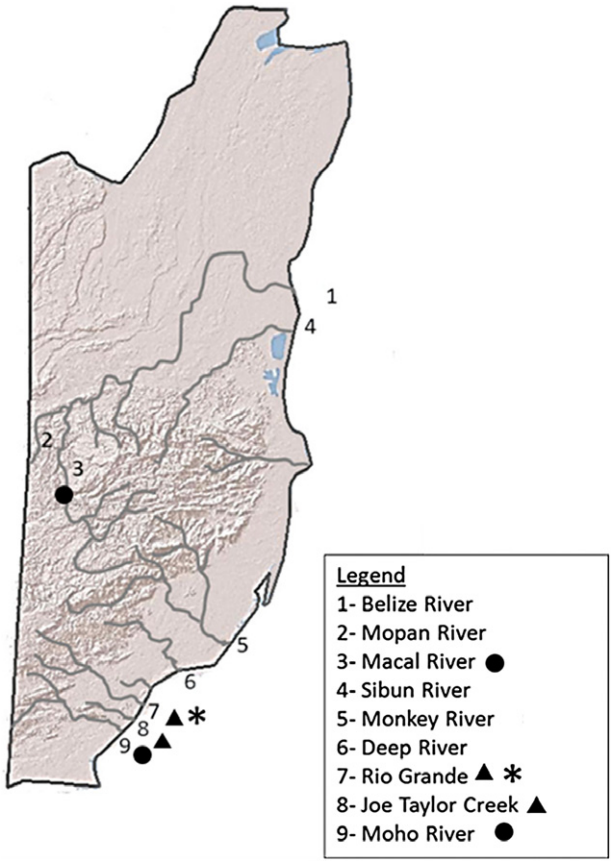

Figure 6. Relief map of Belize. Biogeographical splits reflecting similar history are: ✻, *Belonesox* (present study); ●, *Astyanax* (Strecker *et al*., 2004); and ▲, *Gambusia luma* (Greenfield *et al*., 1982).

The Rio Grande is along the southern edge of the Maya Mountains, a range that is > 248 million years old (Paleozoic) and largely composed of sedimentary rocks called the Santa Rosa Formation (Dixon, 1956; Bateson & Hall, 1977; Weber *et al*., 2006). Having a tolerance for saltwater (Kerfoot *et al*., 2011), *Belonesox* may have been able to disperse with rising flood waters or move through varying conditions to other areas around the Maya Mountains. The foothills of the Maya Mountains become low-lying flood plains moving east to the Caribbean Sea. Intense sea level changes and flooding could have lead to a vicariant event 14.1 Mya where *Belonesox* dispersed south in Toledo, Belize and has subsequently had little to no secondary contact with northern populations. From here, radiation southward into Honduras, Nicaragua and Costa Rica is possible. Although the southern populations remain cut off, connectivity between Belizean and Mexican rivers, namely via the Grijalva-

Usumacinta, which runs through northern Guatemala, could allow gene flow among apparently disjoint populations of the northern clade. Haplotype analyses show this clade is made of three unconnected groups that correspond to these potential areas of intermittent river connectivity. The unconnected nature of the networks show that they have lacked recent gene flow (Fig. 4) but remain more connected to each other than to southern populations.

Other studies have shown biogeographical breaks similar to the results reported in the present study. In a taxonomic study of Belizean *Gambusia*; based on morphological characters, *Gambusia luma* shows a statistically significant shift in the modal number of pectoral fin rays within southern Belize (Greenfield, Greenfield & Wildrick, 1982). The shift occurs between Hot Creek (Rio Grande drainage) and Joe Taylor Creek to the south (Fig. 6). A molecular study of *G. luma* has not been conducted. However, a phylogeographical study of *Astyanax fasciatus* (Characidae) in Central America shows a subdivision between two Belizean populations: one from the north in the Macal River (Belize River drainage) and the other from the south in the Moho River (Fig. 6) (Strecker, Faundez & Wilkins, 2004). Few geological or biogeographical studies have been conducted in Belize. Additional morphological and molecular investigations for many species should help determine the interesting biogeographical nature of southern Belize.

Conclusions

This phylogeographical investigation of *Belonesox* suggests that the subspecies, as described by Hubbs (1936), is not a valid taxon. However, we refrain from officially synonymizing this taxon until a formal morphological study, including the examination of gonopodia, is conducted. The present study recovered a potential, and unexpected, biogeographical break in the Toledo District of southern Belize, which should be investigated with further taxon sampling. Finally, the present study corroborates reports that the Yucatan populations of *Belonesox* are indeed the source of the invasive Florida populations.

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