



Phylogenetic relationships and evolutionary history of the Mesoamerican endemic freshwater fish family Profundulidae (Cyprinodontiformes: Actinopterygii) [☆]

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ABSTRACT

Freshwater fishes of Profundulidae, which until now was composed of two subgenera, represent one of the few extant fish families endemic to Mesoamerica. In this study we investigated the phylogenetic relationships and evolutionary history of the eight recognized extant species (from 37 populations) of Profundulidae using three mitochondrial and one nuclear gene markers (~2.9 Kbp). We applied a Bayesian species delimitation method as a first approach to resolving speciation patterns within Profundulidae considering two different scenarios, eight-species and twelve-species models, obtained in a previous phylogenetic analysis. Based on our results, each of the two subgenera was resolved as monophyletic, with a remarkable molecular divergence of 24.5% for mtDNA and 7.8% for nDNA uncorrected *p* distances, and thus we propose that they correspond to separate genera. Moreover, we propose a conservative taxonomic hypothesis with five species within *Profundulus* and three within *Tlaloc*, although both eight-species and twelve-species models were highly supported by the Bayesian species delimitation analysis, providing additional evidence of higher taxonomic diversity than currently recognized in this family. According to our divergence time estimates, the family originated during the Upper Oligocene 26 Mya, and *Profundulus* and *Tlaloc* diverged in the Upper Oligocene or Lower Miocene about 20 Mya.

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1. Introduction

Profundulidae (Hoedeman and Bronner, 1951), with eight recognized species, represents together with Lacantuniidae (Rodiles-Hernandez et al., 2005), one of the few freshwater fish families endemic to Mesoamerica (Bussing, 1985; Miller, 1966; Myers, 1966). Mesoamerica is regarded as one of the most biologically diverse regions of the world. Its high level of biodiversity has been attributed to its complex geological history and its geographical location, corresponding with a contact zone between the Nearctic and Neotropical biotas. Within Mesoamerica, Profundulidae occurs

on both sides of the Isthmus of Tehuantepec, an area that has been recognized as a biodiversity hotspot for several groups of vertebrates (Escalante et al., 2007; Ferrari, 2000; Huidobro et al., 2006; Marshall and Liebherr, 2000; Mulcahy et al., 2006; Paniagua and Morrone, 2009). Currently, most of the species within Profundulidae exhibit a highly restricted distribution, being confined to basin headwaters of southern Mexico, El Salvador, Honduras and Guatemala in both the Pacific and Atlantic versants (Eschmeyer, 2007; Matamoros et al., 2012; Miller, 1955, 2005; Morcillo, 2004), with the exception of *Profundulus punctatus*, which is able to inhabit the montane and piedmont floodplains and coastal plains. This family is mainly distributed within the Grijalva-Usumacinta basin, an area of endemism (western Guatemala and southern Mexico, Fig. 1), which is characterized by exceptional diversity and where 59% endemism is attributed to freshwater fish fauna (Matamoros et al., 2014).

During recent decades, the family has been subjected to several taxonomic changes, where alternative hypotheses proposed by

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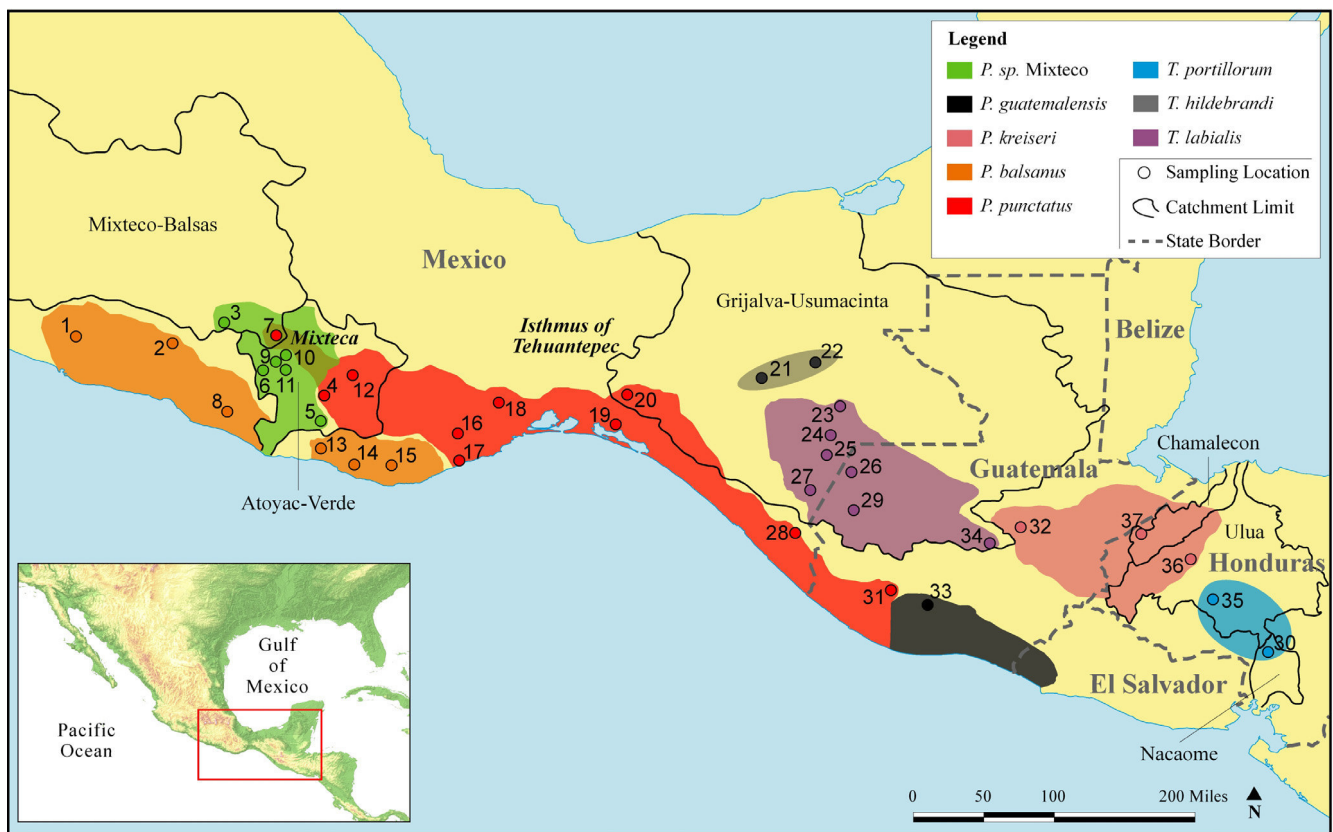


Fig. 1. Sampling localities and species distribution. Map of localities sampled in this study. Numbers correspond to localities shown in [Supplementary data](#), and shaded areas correspond to the 8 species hypothesis proposed for the *Profundulus* and *Tlaloc* subgenera.

morphology and molecular data in some cases have produced contrasting results (Doadrio et al., 1999; Gonzalez-Diaz et al., 2005; Matamoros and Schaefer, 2010; Matamoros et al., 2012; Miller, 1955). These taxonomic changes have arisen in part from difficulties in delimiting species due to a lack of morphological differentiation among some species within the family (i.e. cryptic speciation within (*Profundulus*) *punctatus* species “group”, Miller, 1955), at the same time morphological divergence emerges from ecological adaptations in others (i.e. *Profundulus* (*Tlaloc*) *candalarius* and *Profundulus* (*Tlaloc*) *labialis* (Gonzalez-Diaz et al., 2005; Miller, 1955)). *Profundulidae* represents a feasible model for studying species delimitation, since the family exhibits few species, each with a well-defined distributional range (Fig. 1, Matamoros et al., 2012). According to Miller (2005), the family taxonomy is as follows: one nominal genus, *Profundulus* (Hubbs, 1924) that is divided into two subgenera: *Profundulus* (Hubbs, 1924) comprised of three species, *P. (P.) guatemalensis*, *P. (P.) punctatus* and *P. (P.) oaxacae*, and *Tlaloc* (Alvarez and Carranza, 1951), also including three species, *P. (T.) hildebrandi*, *P. (T.) labialis* and *P. (T.) candaliarius*. Recently, two new species were described: *P. (P.) kreiseri*, which belongs to the *Profundulus* subgenus (Matamoros et al., 2012), and *P. (T.) portillorum*, which belongs to the *Tlaloc* subgenus (Matamoros and Schaefer, 2010). Doadrio et al. (1999) found at least five well-differentiated groups within *P. (P.) cf. punctatus* based on molecular data (allozymes), which could correspond to a higher number of putative species within the subgenus *Profundulus*. Two of them were considered to be new species not yet described: one was known only from a single spring in the Tehuantepec basin and the second extended mainly through springs in the Mixteca region of Mexico (Fig. 1). On the other hand, within the subgenus *Tlaloc*, the validity of the species *P. (T.) candaliarius* has

been questioned because minor and inconsistent morphological differentiation from its sister species *P. (T.) labialis* (Gonzalez-Diaz et al., 2005) and a lack of resolution between these two species using the mtCytb marker suggest recent divergence between the two species (Matamoros et al., 2012).

The present study, which represents the most extensive geographic survey to date for the *Profundulidae* using molecular data, has two main goals. The first one is to formulate a robust phylogenetic hypothesis and the second one is to infer the family evolutionary history. In order to achieve these goals, we performed a molecular phylogenetic reconstruction using three mitochondrial genes (ATP8, ATP6 and ND2) and the S7 nuclear gene. In addition, we carried out a Bayesian relaxed molecular clock analysis to assess the time and mode of diversification in the family.

2. Material and methods

2.1. Taxonomic sampling and tissue collection

A total of 44 specimens were collected from 37 localities situated along most of the known geographic distribution for the family, including samples for all currently recognized species in the family (Fig. 1, Table 1, [Supplementary data 1.1](#)) (Matamoros et al., 2012; Miller, 2005). These localities included populations on the Pacific slope from the Balsas basin (locations 3 and 7, Fig. 1) Mexico, to the Nacaome basin (location 30, Fig. 1), Honduras, and on the Atlantic slope from the Grijalva-Usumacinta basin (Fig. 1), Mexico, to the Ulua basin, Honduras (locations 35 and 36, Fig. 1).

Specimens were sampled by electrofishing and seining under local authorities permission and released onsite. A small

Table 1
Sampling localities and the nomenclature of the locations.

Map	Locality	N	Basin	Location name
1	Rio Huachimil, Tlahuizapa, Guerrero, Mexico	1	Papagayo	Pap2
2	Rio Malinaltepec, Guerrero, Mexico	1	Papagayo	Pap1
3	Rio Grande, Santiago Juxtlahuaca, Oaxaca, Mexico	2	Mixteco-Balsas	Mix2a/2b
4	Spring Sola de Vega, Oaxaca, Mexico.	1	Atoyac	Ato1
5	Spring, San Isidro Sola de Vega, Oaxaca, Mexico	1	Atoyac	Ato6
6	Rio Cucharas, Tuxtla Putla de Guerrero, Oaxaca, Mexico	1	Atoyac	Ato8
7	Rio Chiquito, Tlaxiaco Oaxaca, Mexico	1	Mixteco-Balsas	Mix1
8	Cumiapa river, El Zapote, Santa Maria, Oaxaca, Mexico	1	Quetzala	Que
9	Spring, Aldama, Oaxaca, Mexico	1	Atoyac	Ato5
10	Spring Chalcatongo, Oaxaca, Mexico	1	Atoyac	Chalca
11	Spring, Santiago Yosondua, Oaxaca, Mexico	2	Atoyac	Ato4a/b
12	Rio de las Grutas, San Sebastián de las Grutas Oaxaca, Mexico	1	Atoyac	Ato3
13	Nopala river, Manialtepec, Oaxaca, Mexico	1	Manialtepec	Man
14	Rio Colotepec, San Gabriel Mixtepec, Oaxaca, Mexico	1	Colotepec	Col1
15	Rio Coyul, Santa Maria Huatulco, Oaxaca, Mexico	1	Coyul	Coy
16	Rio Otate, San Miguel Ecatepec, Oaxaca, Mexico	1	Tehuantepec	Tehuantepec
17	Rio Chacalapa river, San Isidro Chacalapa, Oaxaca, Mexico	2	Chacalapa	Chacalapa1/2
18	Rio de los Perros, La Primavera, Oaxaca, Mexico	3	Tehuantepec	Tehuantepec,a/b/c
19	Rio Novillero, Las Minas, Oaxaca, Mexico	1	Novillero	Nov
20	Rio Chiquito, Cintalapa, Chiapas, Mexico	1	Grijalva-Usumacinta	Gri
21	Rio Peje de Oro, San Cristobal de las Casas, Chiapas, Mexico	1	Grijalva-Usumacinta	Chi
22	La Virgen, Ocosingo, Chiapas, Mexico	2	Grijalva-Usumacinta	Vir1/2
23	Spring, El Vergel, Chiapas, Mexico	1	Grijalva-Usumacinta	Ver
24	Rio Grande de Comitán, Emiliano Zapata, Chiapas, Mexico	1	Grijalva-Usumacinta	Gra
25	Rio Chihuahua, Santa Ines, Chiapas, Mexico	1	Grijalva-Usumacinta	Ine
26	Rio Nenton, Finca Chacaj, Huehuetenango, Guatemala	1	Grijalva-Usumacinta	Nen
27	Rio Comalatengo, Nueva Morelia, Comalapa de la Frontera, Chiapas, Mexico	1	Grijalva-Usumacinta	Com1
28	Toliman river, Belisario Dominguez, Chiapas, Mexico	1	Toliman	Tol
29	Selegua river, Colotenango, Huehuetenango, Guatemala	1	Grijalva-Usumacinta	Sel
30	Rio Nacaome, Francisco Morazan, Lepaterique, Honduras	1	Nacaome	H2
31	Rio Bravo, Finca Moca, Suchitepequez, Guatemala	1	Bravo	Bra
32	Cahabon river, Pasmolon, Alta Verapaz, Guatemala	1	Polochic	Pol
33	Rio Blanco, Guacalate, San Miguel Dueñas, Sacatepequez, Guatemala	1	Guacalate	Gua
34	Jeronimo river, San Jeronimo, Baja Verapaz, Guatemala	1	Grijalva-Usumacinta	Jer
35	Canoas river, Siguatepeque, Comayagua, Honduras	1	Ulua	H1
36	Rio Ulua, Santa Barbara, Honduras	1	Ulua	PF1
37	Rio Chamelecon, Santa Barbara, Maculizo, Honduras	2	Chamelecon	PF2/3
	Outgrup, Lebrija, Sevilla, Spain	1	Guadalquivir	Fundulus
	Outgrup, Michoacan, Mexico	1	Lerma	Neotoca

subsample were fin-clipped and individually tagged immediately after euthanasia with an overdose of anaesthetic MS222. The fin clips were preserved in DMSO/EDTA buffer (Seutin et al., 1991) or 95% ethanol. Tissue samples and their associated voucher specimens were deposited in the Museo Nacional de Ciencias Naturales (MNCN-CSIC) in Madrid, Spain, the Ichthyological Collection at the University of Southern Mississippi (USM) and the Ichthyological Collection at the Universidad Nacional Autonoma de Mexico (CNP-UNAM).

Our samples included specimens collected in/or near the type-localities of four currently recognized species of the subgenus *Profundulus*: *P. (P.) oaxacae*, *P. (P.) punctatus*, *P. (P.) guatemalensis* and *P. (P.) kreiseri*, as well as the major synonyms of *P. (P.) punctatus* (Miller, 1955): *P. (P.) balsanus* and *P. (P.) scapularis*. For the subgenus *Tlaloc*, we included topotypes of its four recognized species, *P. (T.) hildebrandi*, *P. (T.) candalaria*, *P. (T.) labialis*, and *P. (T.) portillorum*, as well as the major synonym of *P. (T.) labialis*: *P. (T.) mexicanus* (Miller, 1955) (Supplementary data 1.1). The outgroups selected belong to the families: Fundulidae, *Fundulus heteroclitus* (Linnaeus, 1766) and Goodeidae, *Neotoca bilineata* (Bean, 1887), both sister groups of Profundulidae (Doadrio and Dominguez, 2004; Webb et al., 2004).

2.2. DNA extraction and sequencing of mitochondrial and nuclear DNA

Total cellular DNA was extracted using the BioSprint 15 DNA Blood Kit (Qiagen). For the mtDNA analyses, the entire sequences of the ATP synthase 6 and 8 (ATP8/6, 850 bp) genes and the

complete sequence of the NADH dehydrogenase subunit 2 (ND2, 1047 bp) gene were amplified. We also amplified the first and second introns of the S7 ribosomal protein gene (S7, 998 bp). PCR reactions were performed in 25 µL volume with a final concentration of 0.4 µM of each primer, 0.2 µM of each dNTP, 2 mM MgCl₂, and 1.5 units of Taq DNA polymerase (Biotools). PCR was conducted under the following conditions: 94 °C (2 min), 35 cycles at 94 °C (45 s), region specific T_m °C (1 min), 72 °C (90 ss), and 72 °C (5 min), for most amplifications (for primers see Supplementary data 1.2), except for the S7 gene, in which were followed the PCR conditions described in Chow and Hazama (1998). PCR products were run on 1.0% agarose gels to confirm amplification, PCR products were purified with the EXOSAP-IT PCR Product Clean-Up (Usb). Both strands were sequenced (Supplementary data 1.2) and run on an ABI 3700 DNA automated sequencer (Applied Biosystems, Foster City, CA) at SECUGEN S.L. (Madrid, Spain). Sequence data were submitted to GenBank under the following accession numbers: ND2 from KJ878751 to KJ878789; ATP6 from KJ878663 to KJ878706; ATP8 from KJ878790 to KJ878750; and S7 from KJ878790 to KJ878814 (Supplementary data 1.1).

2.3. Phylogenetic reconstruction

Chromatograms and alignments were visually verified in MEGA5 (Tamura et al., 2011). All our phylogenetic inferences were carried out with two different data sets. The first corresponded to a concatenated matrix of the mitochondrial data, which included ATP8, ATP6 and ND2 genes fragments with separated best-fit

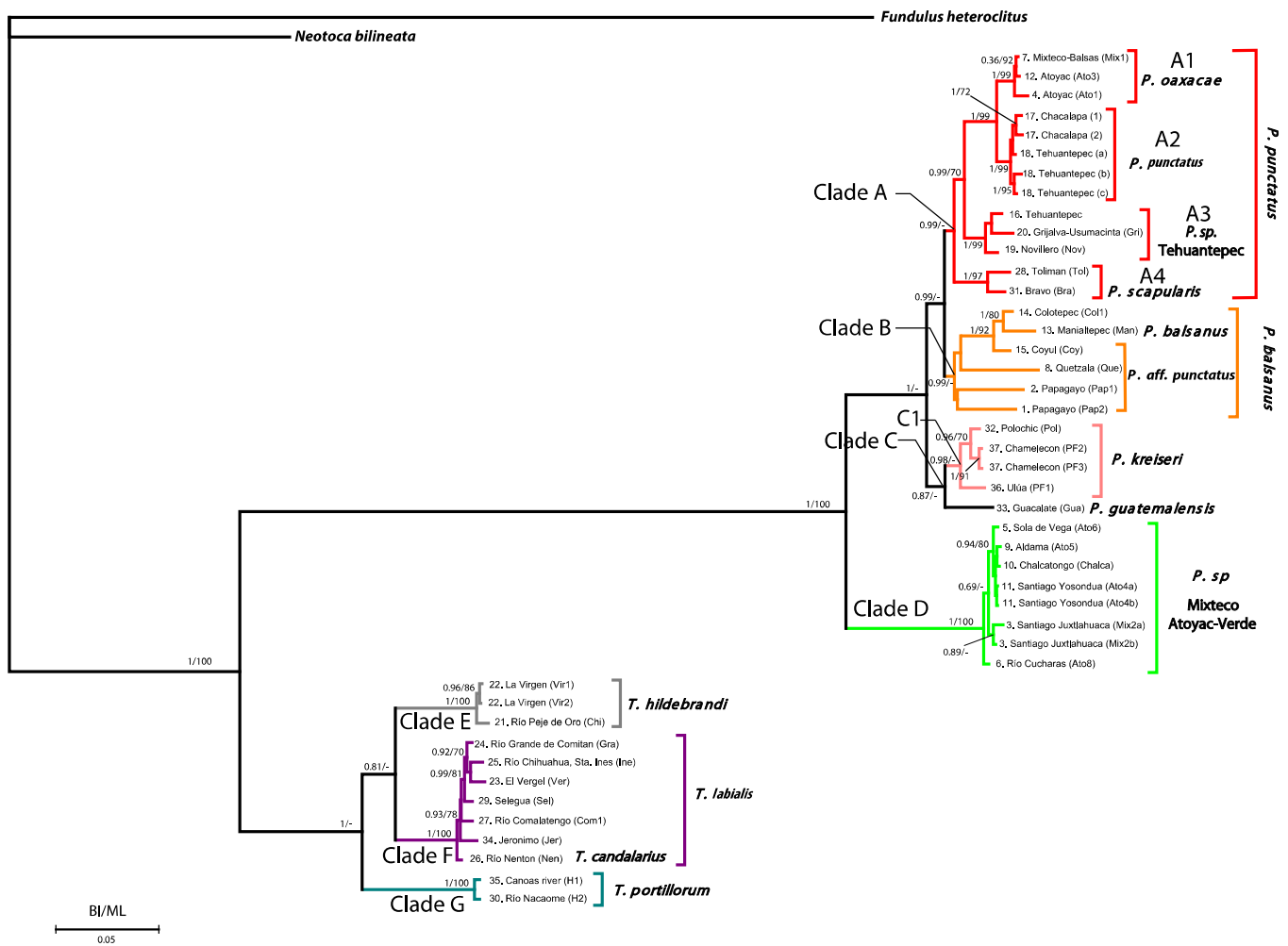


Fig. 2. Topology of mtDNA data matrix. Phylogenetic tree derived from the BI analysis of concatenated mitochondrial matrix (ATP8&6 and ND2) of the Profundulidae populations. Only bootstrap values above 80 and posterior probabilities above 0.8 are shown (values of BI and ML analysis displayed beside each node).

models for each codon position for each gene (mitochondrial data set). The second set corresponded to a concatenated matrix of the three mtDNA genes and the nuclear fragment with a separated best-fit model of evolution for each gene. We identified the evolutionary model of nucleotide substitution for each gene (Supplementary data 1.3) using a corrected Akaike information criterion (AICc) as implemented in the program jModeltest (Posada, 2008). Both uncorrected p distances (D_p ; corresponding to the proportion of different homologous sites), and corrected ML distances (using the substitution models obtained for each codon by gene) were estimated for inter- and intra-taxon divergence at the species and genus levels.

A phylogenetic hypothesis was reconstructed using a Maximum Likelihood (ML) method implemented in PHYML (Guindon and Gascuel, 2003) and AICc-selected parameters. Branch support was calculated using nonparametric bootstrapping (Felsenstein, 1985) with 1000 replicates employing the above evolutionary models.

Bayesian Inference (BI) was carried out using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs were performed using eight Markov chain Monte Carlo (MCMC), with 10 million generations, sampling every thousand generations. The first 1000 trees were discarded as burn-in based on the program Tracer v1.5 (Rambaut and Drummond, 2009). Gene features and parameters employed for each gene using jModeltest (Posada, 2008) are shown in Supplementary data 1.3. These parameters were considered in the partition setting within BI.

2.4. Bayesian species delimitation

A Bayesian multi-locus species delimitation analysis was conducted using the concatenated mtDNA + nDNA dataset in the program BPP 2.2 (Rannala and Yang, 2003; Yang and Rannala, 2010; Yang, 2015). This method uses a species phylogeny represented by a user-specified guide tree, and accommodates lineage sorting due to ancestral polymorphism (Yang and Rannala, 2010). The assumption in the model is that the exchange of migrants is assumed to stop as soon as species diverge. The conducted analysis was species delimitation using a user-specified guide tree (Yang and Rannala, 2010; Rannala and Yang, 2013).

The mtDNA and nDNA were also consistent with the required assumptions to carry out BPP analysis: no recombination within a locus, no migration (gene flow) between species and neutral evolution. The selective neutrality of each mtDNA gene was evaluated using Hudson–Kreitman–Aguadé tests (HKA) (Hudson et al., 1987) in DnaSP v 5.10 (Librado and Rozas, 2009). The nuclear recombination was evaluated using the pairwise homoplasy index (PHI) test (Bruen et al., 2006) as implemented in Splitstree4 (Huson and Bryant, 2006). The BPP analysis was conducted separately by genus in order to avoid the violation of the JC69 mutation model (Jukes and Cantor, 1969), as a consequence of the high divergence between the two genera.

We ran BPP on the concatenated mtDNA + nDNA dataset fully partitioned by gene using the BI and ML topologies obtained in this study as the guide tree. Two species delimitation models were

tested. The first one corresponded to the eight major clades obtained in the BI and ML analysis with mtDNA and with mtDNA + nDNA (Figs. 2 and 3). The second one corresponded to the twelve minor clades obtained in the BI and ML analysis with mtDNA (Fig. 2). We specified a Dirichlet distribution ($\alpha = 2$) to account for variation in mutation rates among loci. A gamma prior (G) was used to specify the population size parameter Θ and root age τ_0 of the species tree considering three different scenarios: (1) Assuming relatively large ancestral population sizes and deep divergences $\Theta \sim G(1, 10)$ and $\tau_0 \sim G(1, 10)$. (2) Assuming relatively small ancestral population sizes and shallow divergences among species: $\Theta \sim G(2, 2000)$ and $\tau_0 \sim G(2, 2000)$, and (3) a conservative combination of priors that could favor models containing fewer species $\Theta \sim G(1, 10)$ and $\tau_0 \sim G(2, 2000)$ (Bagley et al., 2015). Each analysis of 50,000 MCMC generations was run twice from different starting seeds with a burn-in period of 5000 using algorithm 0 (default fine-tuning parameter, $\varepsilon = 2$) and an estimated heridity ($\alpha = 4$, $\beta = 4$); this gave consistent parameter estimates between replicate runs and ESS values >1000 for all parameters. We consider speciation probability values >0.95 as strong support for all speciation events.

2.5. Divergence time estimation

Divergence times among the main mitochondrial clades were estimated using a Bayesian-coalescence approach with BEAST 1.6.1 (Drummond et al., 2006; Drummond and Rambaut, 2007). In this analysis, we considered the mtDNA data set that included the complete ATP 8/6 and a fragment of the ND2 genes (1897 bp) for 44 individuals (Supplementary data 1.1). We applied an uncorrelated lognormal relaxed molecular clock and the Shapiro–Rambaut–Drummond–2006 (SRD06) model of nucleotide substitution to provide better resolution for coding regions. The SRD06 model partitions the nucleotide data by codon position and allows third codon positions to differ from the other two in transition bias, substitution rate and shape of the gamma distribution of rate heterogeneity (Shapiro et al., 2006). The SRD06 model was applied to each gene fragment. We calibrated age estimates using a uniform prior distribution for the mean substitution rate parameter with a mean of 0.8% and lower and upper values of 0.5–1.5% per million years based on what has been reported in other freshwater fish fauna for mitochondrial loci (Concheiro Perez et al., 2007; Doadrio and Dominguez, 2004; Doadrio and Perdices, 2005; Hrbek et al., 2007; Mateos et al., 2002; Murphy et al., 1999; Ornelas-Garcia et al., 2008; Perdices et al., 2005, 2002; Perea et al., 2010).

A test MCMC run with 10 millions generations was performed to optimize the scale factors of the *a priori* function. The final MCMC chain was run twice for 20 million generations, sampling every 1000 generations. We checked for burn-in and convergence and stationarity of the different analyses in Tracer v1.5. The measures of effective sample sizes (ESS) were used to determine the statistical significance of each parameter, and were >200 in most of cases. The results of the independent runs after burn-in were combined in the BEAST module Log Combiner 1.6.1.

3. Results

3.1. Phylogenetic reconstruction

The three mitochondrial (ATP6 and 8 and ND2) and the nuclear gene fragment (S7) gave a total of 2895 bp (1897 mitochondrial and 998 nuclear bp). Members included in the subgenus *Profundulus* had 422 variable sites. Members included in the subgenus *Tlaloc* had 258 variable sites. The mitochondrial data set had 982 variable

sites (52%). The combined data set (mtDNA + nDNA) had 1069 variable sites (37%).

Similar topologies were obtained using both phylogenetic inference methods (ML and BI) and from both the mtDNA and combined data sets (mtDNA and nDNA). The only inconsistency was at the basal nodes of the subgenus *Tlaloc*; in the ML inference the most basal node corresponded to the separation of *P. (T.) hildebrandi* from the rest of species within *Tlaloc*, instead of *P. (T.) portillorum* supported with the BI analyses (Figs. 2 and 3).

Our results support the monophyly of both recognized subgenera, *Profundulus* and *Tlaloc*, between which there is a remarkable genetic divergence, $24.5 \pm 3.4\%$ mtDNA *p* distances and $7.8 \pm 0.3\%$ nDNA *p* distances. Molecular divergence within the subgenus *Profundulus* was $7.5 \pm 10.2\%$ (mtDNA *p* distances) and $1.5 \pm 0.6\%$ (nDNA *p* distances), and within the subgenus *Tlaloc* $5 \pm 3.3\%$ (mtDNA *p* distances) and $1.3 \pm 0.8\%$ (nDNA *p* distances). Similar and lower levels of molecular divergence have been reported between different genera in other Cyprinodontiformes (Doadrio and Dominguez, 2004; Echelle et al., 2006). Based on this remarkable divergence, as well as the high degree of morphological differentiation (Gonzalez-Diaz et al., 2014), we consider *Profundulus* and *Tlaloc* separate genera, and use this nomenclature throughout the rest of the paper.

3.2. Neutrality and recombination

Based on HKA tests, DNA polymorphism levels in the mtDNA data were consistent with expectations of neutral evolution. HKA tests were non-significant for the ATPase 6 ($N = 42$ ingroup sequences, $\chi^2 = 0.008$, $P = 0.9277$), the ATPase8 ($N = 40$ ingroup sequences, $\chi^2 = 0.009$, $P = 0.9277$) and the ND2 ($N = 37$ ingroup sequences, $\chi^2 = 0.007$, $P = 0.9351$) datasets. The PHI test did not find statistically significant evidence for nuclear recombination $p = 0.266$.

3.3. Species delimitation within the *Profundulus* genus

The BI and ML topologies were consistent in the identification of four monophyletic clades within the *Profundulus* genus (A, B C and D, Fig. 2). In general, the gene topologies for mtDNA and concatenated mtDNA + nDNA revealed an allopatric distribution of the four major obtained clades (Figs. 2 and 3).

As it was presented above, within the *Profundulus* genus, two species models were tested, the first assuming five species, which correspond to *P. punctatus*, *P. balsanus*, *P. kreiseri*, *P. guatemalensis* and *P. sp. Mixteco* (Fig. 4b). In the second model, we assume eight species: *P. oaxacae*, *P. punctatus*, *P. sp. Tehuantepec*, *P. scapularis*, *P. balsanus*, *P. kreiseri*, *P. guatemalensis* and *P. sp. Mixteco* (Fig. 4c).

In the two tested models, we observed high speciation probabilities for most of the species assumed in the guide trees (speciation probabilities >0.95). Furthermore, the different prior distributions for Θ and τ_0 do not alter the posterior speciation probabilities. In both the five- and eight-species models, *P. kreiseri* showed the lowest speciation probability values (0.994 and 0.993, respectively).

Within Clade A, four branches (A.1–A.4, Fig. 2) were supported, which correspond to one species in the first model (i.e. *P. punctatus*) or to four species in the second model (i.e. *P. oaxacae*, *P. punctatus*, *P. sp. Tehuantepec* and *P. scapularis*). In the first branch (A.1), we found populations close to the type locality of *P. oaxacae* (location 12, Fig. 1) that are distributed in the headwaters of the Atoyac-Verde and Mixteco basins and in a spring found in the headwaters of the Balsas basin in the Tlaxiaco locality in the Mixteca region (location 7, Fig. 1). Despite the morphological differentiation of this species (Miller, 2005), we found relatively moderate levels of molecular differentiation compared to the remainder of

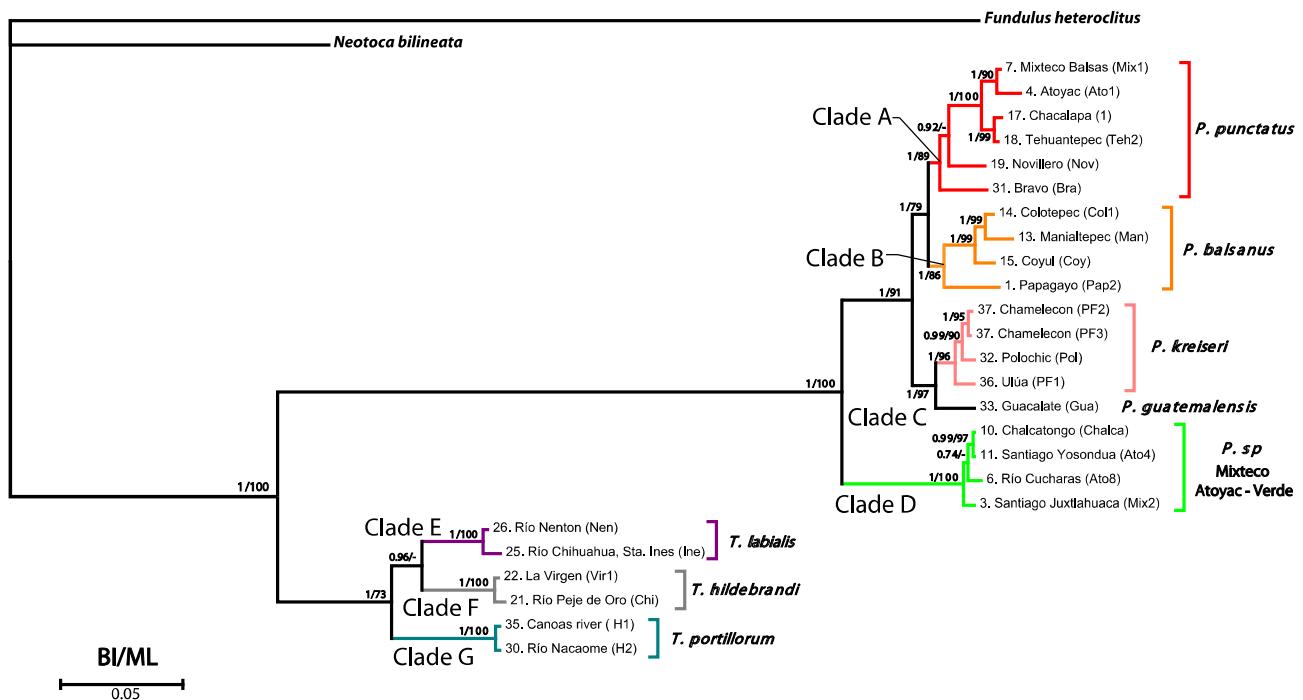


Fig. 3. Topology of mtDNA and nDNA data. Phylogenetic tree derived from the BI analysis of combined data set (ATP8&6 and ND2 and third exon S7). Only bootstrap values above 80 and posterior probabilities above 0.8 are shown (values of BI and ML analysis displayed beside each node).

the *P. punctatus* populations ($D_p = 3.7 \pm 2\%$ mtDNA divergence and $0.9 \pm 0.6\%$ nDNA, [Supplementary data 1.4](#)). In our Bayesian species delimitation, this branch showed high speciation probability values, even in the most conservative model ($P = 1.0$, [Fig. 4c](#)).

The second branch (A.2) included populations of *P. punctatus* from the Tehuantepec (location 18, [Fig. 1](#)) and Chacalapa basins (location 17, [Fig. 1](#)), where the speciation probability values were identical in all models ($P = 1.0$, [Fig. 3](#)). The A.3 branch includes the headwaters of the Grijalva-Usumacinta basin (location 20, [Fig. 1](#)) (Rio Chiquito, Mexico), which drain into the Atlantic slope, and the headwaters of the Novillero (location 19, [Fig. 1](#)) and Tehuantepec (location 16, [Fig. 1](#)) basins on the Pacific slope ([Fig. 2](#)). Finally, A4 included the Rio Bravo basin in Guatemala (location 31, [Fig. 1](#)), which is less than 5 km away from the type locality of *P. scapularis*, and populations from the Toliman basin (location 28, [Fig. 1](#)), both on the Pacific slope. These two last branches both had a speciation probability value of 1.0 for the three different prior distributions for Θ and τ_0 ([Fig. 4b](#) and [c](#)).

Clade B ([Fig. 2](#)) included the type locality of *P. balsanus* (location 2, [Fig. 1](#)) (Papagayo River in Malinaltepec), together with other coastal Pacific basins (location 1, [Fig. 1](#)). Clade B showed high levels of molecular differentiation with respect to its sister Clade A ($D_p = 5.9 \pm 7.2\%$ mtDNA divergence and $1.1 \pm 0.5\%$ nDNA, [Supplementary data 1.5](#)) and speciation probability values of 1.0 in the five and eight-species models ([Fig. 4b](#) and [c](#)).

Clade C ([Fig. 2](#)) included two nominal species, *P. guatemalensis* and the recently described *P. kreiseri* ([Matamoros et al., 2012](#)). The molecular divergence between *P. kreiseri* and *P. guatemalensis* from their type locality is $D_p = 3.1 \pm 0.1\%$ mtDNA divergence and $0.8 \pm 0.1\%$ nDNA ([Supplementary data 1.5](#)). With respect to its distribution, branch C1 included populations of *P. kreiseri* and branch C2 the *P. guatemalensis* population. Our populations of *P. kreiseri* come from the Atlantic slope from Guatemala and Honduras highlands (locations 32, 36 and 37, [Fig. 1](#)). The population of *P. guatemalensis* comes from headwaters from the Guacalate basin (type locality) on the Pacific slope (location 33, [Fig. 1](#)). These two

species had the lowest *P* values in the Bayesian species delimitation analysis, where, in the five-species model, the probability value was 0.994 for the scenario of $\Theta \sim G(1,10)$ and $\tau_0 \sim G(1,10)$. In the eight-species model, we observed similar values for the large ancestral population sizes and deep divergence scenario and for the small ancestral population sizes and shallow divergences among species scenario $\Theta \sim G(2,2000)$ and $\tau_0 \sim G(2,2000)$ with *P* values of 0.993 and 0.994, respectively.

Finally, Clade D ([Fig. 2](#)) is distributed within the springs of the Mixteca region, particularly at the headwaters of the Atoyac-Verde and Mixteco-Balsas basins on the Pacific slope of Mexico (locations 3, 5, 6, 9, 10 and 11, [Fig. 1](#)). These populations, commonly assigned as *P. punctatus*, showed high levels of divergence between co-occurring clades (see [Fig. 1](#), *P. oaxaca* – Clade A –, and *P. sp.* – Clade D –). The molecular divergence of Clade D from the other clades was very high ($D_p = 10.5 \pm 5.9\%$ mtDNA and $D_p = 1.7 \pm 0.2\%$ nDNA Clade D vs. the other three clades of the genus, [Supplementary data 1.5](#)). Despite its high degree of differentiation with other clades, the molecular differentiation within Clade D was very low for both mtDNA and nuclear data ($D_p = 0.4 \pm 0.2\%$ mtDNA divergence and $0.4 \pm 0.4\%$ nDNA). This clade showed speciation probability values of 1.0 for all three different prior distributions for Θ and τ_0 ([Fig. 4b](#) and [c](#)).

3.4. Species delimitation within the *Tlaloc* genus

Within the *Tlaloc* genus, in the BI and ML analysis three clades were obtained (E, F and G), and most of its populations were distributed in the Atlantic slope, mainly at the Grijalva-Usumacinta basin. The only exception was *T. portillorum*, where the only known populations are from Honduras (Rio Nacaome on the Pacific slope, location 30, [Fig. 1](#)) and the Rio Ulua basin on the Atlantic slope (location 35, [Fig. 1](#)), corresponding to the southern limit of the genus. Within this genus, two species models were tested, the first assuming three species, which correspond to *T. portillorum*,

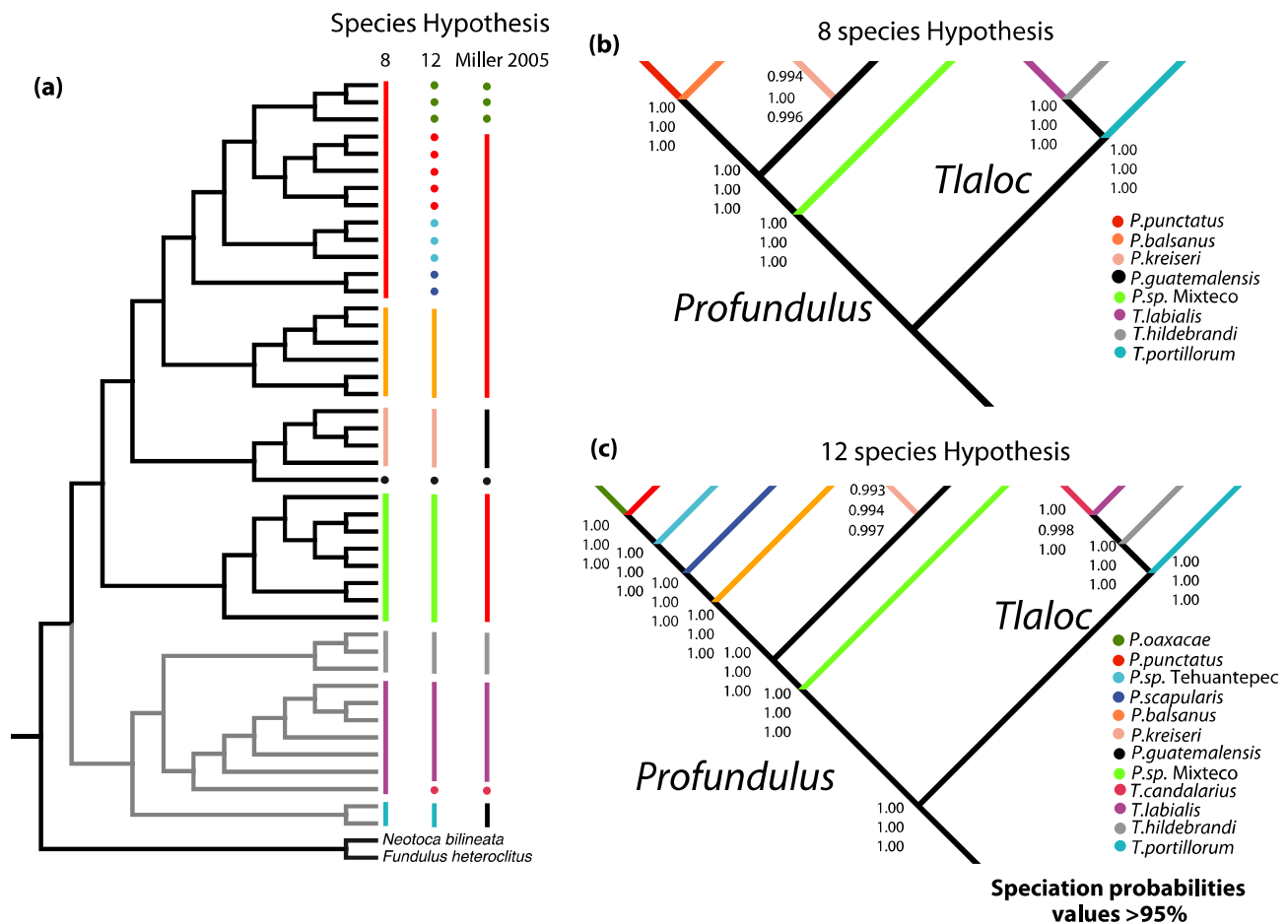


Fig. 4. Alternative species hypothesis. (a) Guide topology based on the BI analysis. Bayesian species delimitation results for Profundulidae assuming eight-species (b) and twelve-species (c) guide trees. The speciation probabilities are provided for each node under each combination of priors for Θ and τ_0 : top, priors $\Theta \sim G(1, 10)$ and $\tau_0 \sim G(1, 10)$; middle, priors $\Theta \sim G(2, 2000)$ and $\tau_0 \sim G(2, 2000)$; bottom, priors $\Theta \sim G(1, 10)$ and $\tau_0 \sim G(2, 2000)$. We consider speciation probability values >0.95 as strong support for speciation event.

T. labialis and *T. hildebrandi*. And the second model, assumed four species, the three aforementioned species plus *T. candaliarius*.

The clades were mostly consistent with the currently recognized species: Clade G with *T. portillorum*, and Clade E with *T. hildebrandi* (Figs. 2–4). However, within clade F we found two nominal species, *T. labialis* and *T. candaliarius*. The genetic levels of differentiation between these two nominal species were low ($D_p = 0.9 \pm 0.2\%$ mtDNA divergence and $0.1 \pm 0.0\%$ nDNA), but contrary to what we expected in our Bayesian delimitation analysis, these two species were supported in the three different prior distributions for Θ and τ_0 although the assumption of relatively small ancestral population sizes and shallow divergences among species ($\Theta \sim G(2, 2000)$ and $\tau_0 \sim G(2, 2000)$) was the only one with the lowest *P* value (0.998, Fig. 4).

When we consider a species model within the *Tlaloc* genus (Fig. 4), we observe high levels of molecular divergences between species, where *T. portillorum* and *T. hildebrandi* showed $8.6 \pm 0.2\%$ mtDNA and $1.8 \pm 0.0\%$ nDNA and *T. portillorum* and *T. labialis* showed $8.3 \pm 0.4\%$ mtDNA and $2.1 \pm 0.0\%$ nDNA (Supplementary data 1.5). These clades had speciation probability values of 1.0 for the three different prior distributions for Θ and τ_0 (Fig. 4).

3.5. Divergence time estimation

The BEAST analysis found a high degree of variation in substitution rate across the studied fragments, as indicated by the marginal

posterior probability of the coefficient of variation of rates: ATP6 mean = 0.9; 95% highest posterior density (HPD) interval = 0.6–1.2, ATP8 mean = 0.7 HPD interval = 0.5–1.1 and the ND2 with the highest mean = 1.2 HPD interval = 1.3 HPD interval = 0.9–1.5. The nodal heights and 95% HPD intervals inferred with BEAST are represented graphically in Fig. 5. The divergence time between *Tlaloc* and *Profundulus* was estimated between the Upper Oligocene and Lower Miocene, (20.2 Mya, 12.5–29.8 Mya). Within the *Profundulus* genus, the isolation of the populations from highlands of the Mixteca region (Clade D) was dated in Upper Miocene (6.8 Mya, 4.1–10.3 Mya). Subsequently, the separation of *P. guatemalensis* from highlands of Guatemala and *P. kreiseri* from the Polochic basin in the Guatemalan highlands from Chamelecon and Ulua basins in Honduras occurred around the Pliocene (3.9 Mya, 2.5–5.6 Mya). Finally, the separation of *P. balsanus* from Papagayo basin and *P. punctatus* populations of the Pacific slope was found around the Pliocene (3.4 Mya, 2.2–4.9 Mya). In the *Tlaloc* genus, the Honduran species, *T. portillorum*, was the first to diverge from the other species of the genus (5.8 Mya, 2.9–9.2 Mya), while the divergence between *T. hildebrandi* and *T. labialis* occurred relatively soon after, in the Pliocene (4.5 Mya, 2.1–7.2 Mya).

4. Discussion

Our results show high divergence between subgenera; the $24.5 \pm 3.4\%$ mtDNA *p* distances and $7.8 \pm 0.3\%$ nDNA *p* distances

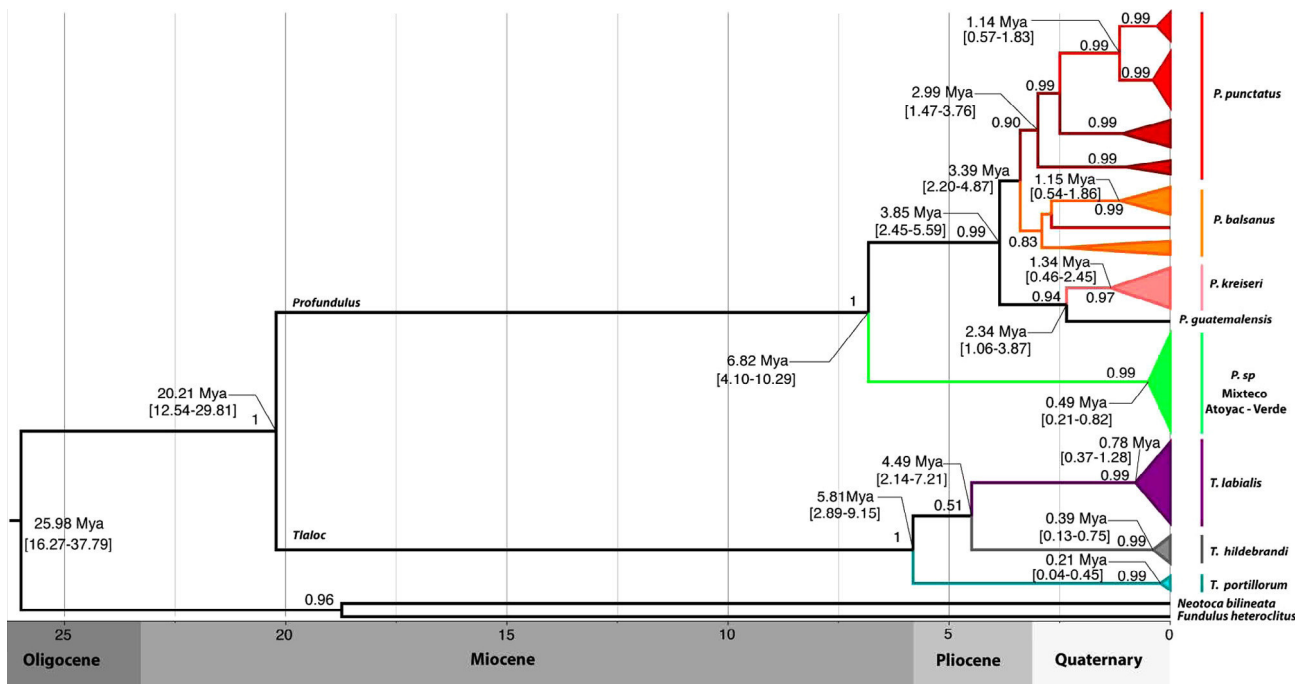


Fig. 5. Molecular Clock. Ultrametric tree based on mtDNA data set (ATP6, ATP8 and ND2), uncorrelated lognormal relaxed molecular clock, using the SRD06 model of nucleotide substitution partitioning the nucleotide data by codon position. Mean divergence times (with 95% highest posterior density intervals for each divergence time in parentheses) are annotated in the main nodes. The scale is in millions of years.

we detected are higher than those reported between other Cyprinodontiformes genera. For example, in their sister Goodeidae family, the divergence reported between *Allophorus* and *Chapalichthys* genera was 6.4% *p* distances for *mtCox1* (Webb et al., 2004) and between *Girardinichthys* and *Skiffia* was 10.5% for *mtCytb* (Doadrio and Dominguez, 2004). Based on both the high degree of molecular differentiation and their morphological distinctiveness (Gonzalez-Díaz et al., 2014; morphological diagnosis in Supplementary data 1.6), we suggest that both subgenera, *Profundulus* and *Tlaloc*, should be considered distinct genera.

Even though the two genera showed an allopatric distribution, the *Profundulus* genus had the widest range, including sections from the Pacific coastal basins in southeastern Mexico (*P. balsanus* in Papagayo basin, locations 1 and 2, Fig. 1) to the Atlantic Ulua basin, in Honduras (*P. kreiseri*) (location 36, Fig. 1). The *Tlaloc* genus exhibited a distribution restricted to the upper part of the Grijalva-Usumancinta, Mexico (Fig. 1), and the Ulua River in Honduras (location 35, Fig. 1).

In terms of species diversity, we also observed differences between genera, with *Profundulus* having higher species diversity than *Tlaloc* (Fig. 4). In both scenarios (eight-species or twelve-species) the most divergent species within the *Profundulus* genus corresponded to *P. sp. Mixteco* (Clade D), which is distributed in the Mixteca region (Figs. 1 and 2), with $9.35 \pm 1.5\%$ mtDNA *p* distances and $1.8 \pm 0.15\%$ nDNA *p* distances with the other species within *Profundulus*. This result is consistent with the previous observations of Doadrio et al. (1999) using allozyme data, where the Mixteca region (Fig. 1) was differentiated from the rest of the species within the *Profundulus* genus. For instance, in *Poeciliopsis*, divergence between species reported is 10% mtDNA (Mateos, 2005) and in the Goodeidae, divergence between *Girardinichthys spp.* and *Skiffia spp.*, is reported at 10% of mtDNA *p* distances (Doadrio and Dominguez, 2004). Further studies are needed to determine the taxonomic status of Mixteca region populations.

Particularly, in the eight-species scenario (Fig. 4b), five species correspond to the *Profundulus* genus: *P. punctatus*, *P. balsanus*,

P. kreiseri, *P. guatemalensis* and *P. sp. Mixteco*, with a mean divergence between species of $6.35 \pm 2.8\%$ mtDNA *p* distances and $1.58 \pm 0.37\%$ nDNA *p* distances. All five aforementioned species showed high levels of Bayesian speciation probability.

In the twelve-species scenario, four species within *P. aff. punctatus* were supported: *P. oaxacae sensu Miller* (2005), from the upper part of the Mixteca region (Fig. 1), with a relatively high divergence from the rest ($Dp = 3.7 \pm 1.6\%$ mtDNA and $Dp = 0.9 \pm 0.6\%$ nDNA), and three well-differentiated branches: A2 corresponding to *P. punctatus*, A3 to *P. sp. Tehuantepec* which could previously detected using allozymic data (Doadrio et al., 1999) and A4 that included the type locality of *P. scapularis*, synonym of *P. punctatus* (Figs. 2–4). Moreover, these four branches within *P. aff. punctatus* were supported with high Bayesian speciation probabilities ($P > 0.95$) independent of the prior distribution of Θ and τ_0 (Fig. 4). Although further analyses are needed to determine the taxonomic status of these additional branches, we consider that they could correspond to valid taxa.

Additionally, in both the eight-species and twelve-species scenarios, *P. balsanus* was supported as a valid taxon, where the species distribution range included the Pacific coastal tributaries from Papagayo (Guerrero) (location 1, Fig. 1) to Rio Coyul (Oaxaca) (location 15, Fig. 1), including the type locality of *P. balsanus* (Malinaltepec, Papagayo basin) (location 2, Fig. 1). In a previous analysis, Miller (1955) also found *P. balsanus* to be a valid form, however he synonymized it with *P. punctatus* due to the uncertainty in the meristic characters, as well as the conflict with the identification of type locality. The molecular divergence between *P. punctatus* (Clade A) and *P. balsanus* (Clade B) was 5.9% mtDNA *p* distances and 1.4% nDNA *p* distances. Therefore, we suggest that *P. balsanus* should be recognized as a valid taxon, with a distribution range including northern Pacific coastal tributaries (Fig. 1), as suggested in the original species description (see Miller's, 1955 and references within).

Finally, based on our results *P. kreiseri* presents a wider distribution range than formerly proposed (Matamoros et al., 2012); from

the Cahabon River in the Polochic basin in Guatemala (location 32, Fig. 1) to the Ulua basin, Honduras, (location 36, Fig. 1) both on the Atlantic slope.

Within the *Tlaloc* genus, both taxonomic scenarios were very consistent. In the eight-species scenario three species were supported (Fig. 4b): *T. hildebrandi*, *T. labialis* and *T. portillorum*, all with Bayesian speciation probabilities equal to 1.00, independent of the priors distributions of Θ and τ_0 (Fig. 4). The mean divergence between the *Tlaloc* species was $7.8 \pm 1.13\%$ mtDNA *p* distances and $1.6 \pm 0.62\%$ nDNA *p* distances. Though, in the twelve-species scenario, *T. candalaria* was considered a valid taxon (Fig. 4c), showing a mean divergence of 0.9 ± 0.2 mtDNA *p* distances from its sister taxon *T. labialis* (Supplementary data 1.4). Based on our data (Bayesian speciation probabilities over 95%) we suggest that *T. candalaria* could effectively be the product of a very recent speciation event (Fig. 4c), and further studies of both it and its sister species *P. labialis* could help to elucidate the degree of ecological divergence between them.

Although both the eight-species and twelve-species models were highly supported, we propose the eight-species one as conservative taxonomic hypothesis according to our findings and current morphological evidences: within the *Profundulus* genus, *P. punctatus*, *P. guatemalensis*, *P. kreiseri*, *P. balsanus* and *P. sp.* Mixteco; within *Tlaloc* genus, *T. labialis*, *T. hildebrandi* and *T. portillorum*. Both models provide additional evidence of greater taxonomic diversity than originally recognized in this family and the importance of carry out more studies to solve the taxonomy of the genera, particularly in the *Profundulus* genus.

Related to evolutionary history, the results of this study suggest that the two genera of Profundulidae diverged during the early Miocene 20.2 Mya (CI = 12.54–29.81 Mya) and there is a temporal congruence with the mountain uplift that divided the Atlantic and Pacific slopes (Doadrio et al., 1999). Within *Profundulus* genus, the isolation of the populations from highlands of the Mixteca region (Clade D) was dated in Upper Miocene (6.8 Mya, 4.1–10.3 Mya). Subsequently, the separation of *P. guatemalensis* from the “*balsanus*” group of *Profundulus*, that appears southwestern of the Chacalapa fault, occurred around the Pliocene (3.9 Mya, 2.5–5.6 Mya). In the study of the *Profundulus* genus using allozymes, Doadrio et al. (1999) stated that the Tehuantepec Isthmus was a recent barrier that divided the *Profundulus* populations. Within *Profundulus* genus, the presence of sporadic populations in the headstreams of some Atlantic Rivers must be interpreted as a recent dispersion, probably during the Pleistocene (Doadrio et al., 1999). Within the *Tlaloc* genus, *T. portillorum* diverged from *T. labialis* and *T. hildebrandi* during the Pliocene.

Miller (1955) suggested that both genera diversified in allopatry, followed by a secondary contact where *Profundulus* species were, in most cases, distributed on the Pacific slope, while the *Tlaloc* species were distributed on the Atlantic slope. We suggest that the presence of the *Profundulus* genus on the Atlantic versant, such as the occurrence of *P. punctatus* in the headwater of Papaloapan basin in Veracruz Mexico (Doadrio com. pers.) and in the headwaters of Grijalva–Usumacinta basin (location 20, Fig. 1) and *P. kreiseri* in the Cahabon – Polochic drainage (location 32, Fig. 1), could be the result of river captures. Moreover, *P. kreiseri* inhabits in the Atlantic slope at the upper reaches of the Chamelecon (location 37, Fig. 1), Motagua and Ulua (location 36, Fig. 1) drainages, while at the Pacific this species has been found at the Lempa river in El Salvador (McMahan et al., 2013), where some authors have suggested that river captures could have occurred for other groups of fishes (e.g. Cahabon – Polochic drainage in *Astyanax* genus (Ornelas-García et al., 2008)).

Furthermore, Miller (1955) suggested that the distribution of *P. punctatus* on the Atlantic versant could be the result of river captures at the city of La Antigua, Guatemala. Further studies

comparing multiple fish groups in the region could shed more light on the hydrological history of the region.

Finally, in accordance with our molecular dates, we observed that the major cladogenetic events could have occurred during the three periods of Neotropical Diversification (Rull, 2011). Profundulidae could be as ancient as 26 Mya old (37–16 Mya), when the *Profundulus* and *Tlaloc* subgenera showed mostly allopatric distributions (Figs. 1 and 5). This date would coincide with the first episode of diversification (Lower–Middle Miocene, 30–12 Mya) when occurred a high tectonic activity in the region. The second period corresponds to the Upper Miocene – Lower Pliocene (14–10 Mya), followed by the most recent diversification event that could be the result of Pleistocene climatic changes, which occurred during the Quaternary (<2 Mya).

5. Conclusions

Profundulus and *Tlaloc* are supported as monophyletic and we propose that they are distinct genera rather than subgenera within Profundulidae. Bayesian species delimitation in the family was consistent independently of priors used. We applied a Bayesian species delimitation method to suggest a first approach to describing the speciation patterns within Profundulidae taking into account two different scenarios, one considering the eight major clades obtained in the BI and ML analysis with mtDNA and nDNA, and a second model with twelve putative species (minor clades in the aforementioned BI analysis). We propose a conservative taxonomic hypothesis according to our findings and current morphological evidences: five species within the *Profundulus* genus, *P. punctatus*, *P. guatemalensis*, *P. kreiseri*, *P. balsanus* and *P. sp.* Mixteco and three species within *Tlaloc* genus, *T. labialis*, *T. hildebrandi* and *T. portillorum*. Although both the eight-species and twelve-species models were highly supported, they provide additional evidence for the existence of a greater taxonomic diversity than the originally recognized in this family and the importance of carry out more studies to solve the taxonomy of the genera, particularly in the *Profundulus* genus.

The Profundulidae, which originated during the Upper Oligocene, presented two major pulses of diversification that could coincide with diversification events in other groups of freshwater fish. The first, during the Lower Miocene, could be related to volcanic activity of the region, followed by a more recent event in the Quaternary, coinciding with Pleistocene climatic fluctuations. The difficulty in establishing species limits in Profundulidae, mainly in *Profundulus* genus, is a reflection of the complex geological history of Nuclear Central America, chiefly in the Isthmus of Tehuantepec area.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jympev.2015.09.002>.

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