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HOW LONG DOES EVOLUTION OF THE TROGLOMORPHIC FORM TAKE? ESTIMATING DIVERGENCE TIMES IN ASTYANAX MEXICANUS

KAKO DOLGO TRAJA EVOLUCIJA TROGLOMORFNIH OBLIK? OCENJEVANJE DIVERGENČNIH ČASOV PRI ASTYANAX MEXICANUS

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Features including colonization routes (stream capture) and the existence of both epigean and cave-adapted hypogean populations make *Astyanax mexicanus* an attractive system for investigating the subterranean evolutionary time necessary for acquisition of the troglomorphic form. Using published sequences, we have estimated divergence times for A. mexicanus using: 1) two different population-level mitochondrial datasets (cytochrome b and NADH dehydrogenase 2) with both strict and relaxed molecular clock methods, and 2) broad phylogenetic approaches combining fossil calibrations and with four nuclear (recombination activating gene, seven in absentia, forkhead, and α-tropomyosin) and two mitochondrial (16S rDNA and cytochrome b) genes. Using these datasets, we have estimated divergence times for three events in the evolutionary history of troglomorphic *A. mexicanus* populations. First, divergence among cave haplotypes occurred in the Pleistocene, possibly correlating with fluctuating water levels allowing the colonization and subsequent isolation of new subterranean habitats. Second, in one lineage, *A. mexicanus* cave populations experienced introgressive hybridization events with recent surface populations (0.26-2.0 Ma), possibly also correlated with Pleistocene events. Finally, using divergence times from surface populations in the lineage without evidence of introgression as an estimate, the acquisition of the troglomorphic form in *A. mexicanus* is younger than 2.2 (fossil calibration estimates) – 5.2 (cytb estimate) Ma (Pliocene).

Key words: *Astyanax mexicanus,* divergence time, troglomorphy, subterranean, evolution.

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Megan L. Porter, Katharina Dittmar & Marcos Pérez-Losada: Kako dolgo traja evolucija troglomorfnih oblik? Ocenjevanje divergenčnih časov pri Astyanax mexicanus

Značilnosti, ki vključujejo tudi kolonizacijske poti in obstoj tako epigejičnih kot hipogejičnih populacij vrste *Astyanax mexicanus,* ji omogočajo, da predstavlja zanimiv sistem za proučevanje evolucije in časa, potrebnega za razvoj podzemeljskih troglomorfnih oblik. Za *A. mexicanus* smo na podlagi že objavljenih sekvenc ocenili divergenčni čas ob uporabi: 1) dveh različnih populacijskih mitohondrialnih podatkovnih baz (citokrom b in NADH dehidrogenaze 2), obe z natančno in sproščeno metodo molekularne ure, in 2) razširjenega filogenetskega pristopa v kombinaciji s fosilno kalibracijo ter štirimi jedrnimi geni (rekombinacijski aktivacijski gen, »forkhead kontrolni gen« in α-tropomiozin) in dvema mitohondrialnima genoma (16S rDNA in citokrom b). Ob uporabi navedenih podatkovnih baz smo ocenili divergenčni čas za tri dogodke v zgodovini razvoja troglomorfnih populacij *A. mexicanus.* Prvič, razhajanje med podzemeljskimi haplotipi se je zgodilo v Pleistocenu, verjetno v odvisnosti od nihanja vode, ki je omogočilo kolonizacijo in posledično izolacijo v novih podzemeljskih habitatih. Drugič, verjetno je v povezavi s pleistocenskimi dogodki pri eni liniji podzemeljskih populacij *A. mexicanus* prišlo do introgresivne hibridizacije s takratnimi površinskimi populacijami (0.26-2.0 Ma). Z uporabo divergenčnega časa površinskih populacij tistih linij, ki ne kažejo introgresije ocenjujemo, da je troglomorfna oblika *A. mexicanus* mlajša od 2,2 (ocene fosilne kalibracije) do 5,2 milijona let (cytb ocena) (Pliocen).

Ključne besede: *Astyanax mexicanus,* divergenčni čas, troglomorfizem, podzemlje, speleobiologija, evolucija.

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INTRODUCTION

Understanding the evolution of the cave form has fascinated biologists interested in subterranean faunas since Darwin. Termed 'troglomorphy', the suite of progressive and regressive characters associated with cavernicolous animals can be observed in the worldwide convergence of form found in the cave environment, exhibited in similar structural, functional, and behavioral changes across diverse taxonomic groups. Much of the debate over troglomorphy has centered on the evolutionary mechanisms responsible for character regression, generally argued to be either neutral mutation or natural selection. Several studies, *(Gammarus minus* - Culver *et al.*, 1995; *Astyanax mexicanus* – Jeffery, 2005) have shown eye degeneration is the result of selection, and, in the case of *A. mexicanus,* is caused by the pleiotropic effects of natural selection for constructive traits. Another, less studied, aspect of understanding troglomorphy is the evolutionary time required to gain the cave form. Because it is generally difficult to pinpoint the time of subterranean colonization and isolation from surface ancestors, few troglomorphic species offer the opportunity for quantitative estimates of the evolutionary time spent in the subterranean realm. Therefore, the time of cave adaptation is thought of in relative terms, where the degree of eye and pigment reduction indicates the period of cavernicolous evolution and therefore the relative phylogenetic age of each species (Aden, 2005).

In evolutionary studies of cave adaptation, *Astyanax mexicanus* has become a model system (Jeffery, 2001). The advantageous features of *A. mexicanus* as a model system include the existence of both surface and troglomorphic cavefish populations, with several cave fish populations having evolved constructive and regressive changes independently (Jeffery, 2001). Furthermore, since the discovery of the species in 1936 (Hubbs & Innes, 1936), there has been an extensive amount of research devoted to characterizing developmental, phylogenetic, taxonomic, and biogeographic aspects of the species (Jeffery, 2001; Mitchell *et al.*, 1977; Wiley & Mitchell, 1971;). In terms of being a model system for understanding the evolution of the troglomorphic form, *A. mexicanus* has at least one additional favorable attribute. The primary mode of *A. mexicanus* subterranean colonization is via stream capture, with most of the captured surface drainages no longer supporting epigean populations (Mitchell *et al.*, 1977). These captures provide discrete colonization events correlated with divergence time from surface populations and therefore with the time of subterranean evolution.

Molecular studies that have looked at *A. mexicanus* phylogeography indicate that at least two independent invasions of surface *Astyanax* have occurred (Dowling *et al.*, 2002a; Strecker *et al.*, 2003, 2004). These two distinct *A. mexicanus* genetic lineages consist of cave fish from La Cueva Chica, La Cueva de El Pachón, El Sótano de Yerbaniz, El Sótano de Molino, El Sótano de Pichijumo, and La Cueva del Río Subterráneo (lineage A) and from La Cueva de los Sabinos, El Sótano de la Tinaja, La Cueva de la Curva, and El Sótano de Las Piedras (Lineage B) with different evolutionary histories - Lineage A clusters with closely related epigean populations while lineage B has no closely related epigean counterparts. The close association of Lineage A to epigean populations (as estimated by mitochondrial markers) is thought to be the result of either recent subterranean colonization or reflect recent introgressive hybridization with surface populations, while lineage B is considered to be a more ancient colonization event from surface populations that are extinct in the region (Dowling *et al.*, 2002a; Strecker *et al.*, 2004). Although the evolutionary histories of different hypogean A. mexicanus populations are complex, the two lineages offer the unique opportunity to estimate the divergence time required for the evolution of the troglomorphic form based on discrete times of colonization and the previous molecular studies of their phylogeography. At least one other study has estimated lineage ages in *A. mexicanus* populations; however, this study was based on a single gene molecular clock estimate and did not specifically estimate the divergence times of the cave populations (Strecker *et al.*, 2003). Here we use three different sets of publicly available sequence data and known fossil calibrations and apply multiple phylogenetic approaches to estimate the age of cave colonization and stream capture events, and to provide an estimate of the time necessary to acquire the troglomorphic form in *A. mexicanus.*

METHODS

Sequence Data

Data were acquired from Genbank (http://www.ncbi.nlm.nih.gov/) from previously published studies of *A. mexicanus* and characiform fishes (Tab. 1). These studies provided three different datasets, consisting of: 1) population-level haplotype datasets for the mitochondrial cytochrome b (cytb; Strecker *et al.*, 2004) and NADH dehydrogenase 2 (ND2; Dowling *et al.*, 2002a) genes, and 2) a species-level dataset of four nuclear (recombination activating gene – RAG2; seven in absentia – sina; forkhead – fkh; and α-tropomyosin - trop) and two mitochondrial genes (16S rDNA and cyt*b*) from representatives within the Otophysi (Calcagnotto *et al.*, 2005). Divergence times from all three data sets were estimated and compared.

Species-level Phylogenetic Analyses

The species-level dataset included selected Otophysi, Characiformes, and Characidae sequences (see Tab. 1), and was analyzed using Anotophysi species as outgroups. Representative *A. mexicanus* cyt*b* haplotype sequences from the Strecker *et al.,* (2004) study were included in the dataset of characiform species to estimate divergence times based on fossil calibrations for comparison with population-based estimates utilizing substitution rates. Alignments of protein-coding regions were trivial and were accomplished using amino acid translations. Sequences of the trop gene spanned an intron, which was removed due to significant length variation (70-836 bp) leading to ambiguous alignments. The alignment of the 16s rDNA gene was generated using the E-INS-i accuracy-oriented strategy of MAFFT v.5 (Katoh *et al.,* 2005). All of the individually aligned genes were then concatenated to form a single dataset consisting 3770bp in length. The concatenated dataset was analyzed with PAUP* 4.0b10 (Swofford, 2000) using maximum parsimony and implementing the parsimony ratchet method (Nixon, 1999) using a batch file generated by PAUPRat with the default parameters for 5000 replicates (Sikes & Lewis, 2001).

Divergence time estimation

Population analysis. Dates of divergence were inferred for A. mexicanus lineage A and B cave fish populations using the cyt*b* and ND2 datasets with BEASTv1.4 (Drummond & Rambaut, 2003). Because the cytb and ND2 haplotype datasets were generated from different studies, they cannot be combined. Therefore, each dataset was used to independently estimate the divergence times of the *A. mexicanus* cave-adapted haplotype sequences. Each dataset was analyzed using both strict and relaxed clock models (Drummond *et al.,* 2006) tested under constant and skyline models of population growth. As part of BEAST divergence time estimation, either a calibration point (fossil or geologic) or a gene-specific substitution rate is required. Because there are no geologic dates corresponding to *A. mexicanus* populations invading subterranean systems, substitution rates were used. For each gene, the range of substitution rates calculated for other freshwater fish were used. For cyt*b*, mean substitution rates ranged from 0.005 to 0.017 substitutions/site/million year (my) (Bermingham *et al.*, 1997; Burridge *et al.*, 2006; Dowling *et al.,* 2002b; Perdices & Doadrio, 2001; Sivasundar *et al.,* 2001; Zardoya & Doadrio, 1999) and for ND2 mean substitution rates ranged from 0.011 to 0.026 substitutions/site/my (Near *et al.,* 2003; Mateos, 2005). These independent rates were used to calibrate the rate of evolution of our datasets by either fixing the rate to the lowest and highest value estimated for each gene or using strong prior distributions on the substitution rates. Two independent MCMC analyses $2x10^7$ steps long were performed sampling every $2{,}000^{th}$ generation, with a burn-in of $2x10^6$ generations. All the Bayesian MCMC output generated by BEAST was analyzed in Tracer v1.3 (Drummond & Rambaut, 2003).

Likelihood-based AHRS method. We used the likelihood heuristic rate-smoothing algorithm of (Yang, 2004) as implemented in PAML3.14 (Yang, 2001). Sequence data were analyzed using the F84+Γ model. Branches at each locus were classified into four rate groups according to their estimated rates. The oldest known fossil representatives of major lineages within the Ostariophysi are well established in recent literature (see Briggs, 2005 and references therein), and have been used in recent studies estimating molecular-based divergence times of Otocephalan clades (Peng *et al.,* 2006). These fossil representatives were used as calibration points for the AHRS divergence time analysis (Fig. 1, Tab. 2,). Fossil calibrations were accommodated as fixed ages and mapped to the basal node of the clade of interest. Given that most fossils are dated to an age range, the minimum and maximum ages of each fossil were used for divergence time estimations under separate analyses. Fossil dates were determined using the 1999 GSA Geologic Time Scale.

Tab. 1: Taxonomy, gene data, and Genbank accession numbers for sequences used in Characiformes phylogeny reconstruction. Abbreviations of mitochondrial gene sequences: 16S = 16S rDNA, cytb = cytochrome b; abbreviations for nuclear gene sequences: fkh = forkhead, RAG2 = recombination activating gene, sina = seven in absentia, trop = α-tropomyosin.

	16S	cytb	fkh	RAG2	sina	trop
Anotophysi (outgroup)						
Chanidae						
Chanos chanos	NC004693	NC004693	---	---	---	---
Gonorynchidae						
Gonorynchus greyi	NC004702	NC004702	---	---	---	---
Kneriidae						
Cromeria nilotica	NC007881	NC007881	---	---	---	---
Parakneria cameronensis	NC007891	NC007891	---	---	---	---
Otophysi (ingroup)						
CHARACIFORMES						
Anostomidae						
Leporinus sp.	AY788044	AY791416	AY817370	AY804095	AY790102	AY817252
Chilodontidae						
Chilodus punctatus	AY787997	---	AY817325	---	AY790056	AY817215
Prochilodontidae						
Prochilodus nigricans	AY788075	AY791437	AY817400	AY804120	AY790133	AY817278
Hemiodontidae						
Hemiodus gracilis	AY788027	AY791405	AY817353	AY804084	AY790086	AY817240
Parodontidae						
Parodon sp.	AY788065	AY791427	AY817390	AY804110	AY790123	AY817269
Serrasalmidae						
Colossoma macropomum	AY788000	AY791386	AY817328	AY804061	AY790059	AY817218
Cynodontidae						
Hydrolycus pectoralis	AY788033	---	AY817359	AY804088	AY790091	AY817244
Characidae						
Acestrorhynchus sp.	AY787956	AY791353	AY817288	AY804026	AY790014	AY817181
Aphyocheirodon sp.	AY787966	AY791363	AY817298	AY804031	AY790025	---
Astyanacinus sp.1	AY787969	AY791365	AY817301	AY804033	AY790028	AY817190
Astyanacinus sp.2	AY787987	---	AY817317	AY804051	AY790046	AY817209
Astyanax bimaculatus	AY787955	---	AY817287	AY804025	AY790013	AY817180
Astyanax mexicanus (Brazil)	---	AY177206	---	---	---	---
Astyanax mexicanus (haplotype AB)	--	AY639041	--	--	--	--
Astyanax mexicanus (haplotype AL)	--	AY639051	--	--	--	--
Astyanax mexicanus (haplotype EA)	--	AY639075	--	--	--	--
Astyanax mexicanus (haplotype FA)	--	AY639084	--	--	--	--
Astyanax mexicanus (haplotype GA)	--	AY639089	--	--	--	--
Astyanax mexicanus (haplotype GB)	--	AY639090	--	--	--	--
Astyanax scabripinis	AY787967	---	AY817299	---	AY790026	AY817188
Brycon hilarii	AY787976	AY791370	AY817307	AY804040	AY790035	AY817198
Bryconamericus diaphanus	AY787984	AY791375	AY817314	AY804048	AY790043	AY817206
Bryconops sp.	AY787985	AY791376	AY817315	AY804049	AY790044	AY817207
Chalceus erythrurus	AY787990	AY791379	AY817320	AY804053	AY790049	AY817211
Chalceus macrolepidotus	AY787999	AY791385	AY817327	AY804060	AY790058	AY817217
Cheirodon sp.	AY787995	AY791382	AY817324	AY804057	AY790054	---
Cheirodontops sp.	AY787996	AY791383	---	AY804058	AY790055	---
Creagrutus sp.	AY788001	---	---	AY804062	AY790060	AY817219
Exodon paradoxus	AY788013	AY791397	AY817340	AY804072	AY790072	AY817227
Gephyrocharax sp.	AY788014	AY791398	AY817341	AY804073	AY790073	AY817228
Hemibrycon beni	AY788020	AY791402	AY817346	AY804079	AY790079	AY817234
Hemigrammus bleheri	AY788017	---	AY817343	AY804076	AY790076	AY817231

	16S	cytb	fkh	RAG2	sina	trop
Hemigrammus erythrozonus	AY788023	---	AY817349	AY804081	AY790082	AY817236
Hemigrammus rodwayi	AY788034	---	AY817360	AY804089	AY790092	AY817245
Hyphessobrycon eques	AY788022	---	AY817348	AY804080	AY790081	AY817235
Inpaichthys kerri	AY788039	---	AY817365	AY804093	AY790097	AY817248
Knodus sp.	AY788041	AY791414	AY817367	AY804094	AY790099	AY817249
Moenkhausia sanctaphilomenae	AY788054	---	---	AY804104	AY790112	AY817261
Mimagoniates lateralis	AY788051	AY791420	AY817377	AY804101	AY790109	AY817259
Prodontocharax sp.	AY788064	AY791426	AY817389	AY804109	AY790122	---
Roeboides sp.	AY787994	AY791381	AY817323	AY804056	AY790053	AY817214
Salminus maxillosus	AY788080	AY791438	AY817405	AY804124	AY790137	AY817282
Triportheus angulatus	AY788082	---	AY817407	AY804125	AY790139	AY817283
Ctenolucidae						
Ctenolucius hujeta	AY787998	AY791384	AY817326	AY804059	AY790057	AY817216
Lebiasinidae						
Nannostomus beckfordi	AY788059	---	AY817384	---	AY790117	AY817265
Crenuchidae						
Characidium fasciatum	AY787992	AY791380	AY817322	AY804055	AY790051	AY817213
Erythrinidae						
Hoplias sp.	AY788031	AY791409	AY817357	AY804087	AY790090	AY817242
Alestidae						
Arnoldichthys spilopterus	AY787968	AY791364	AY817300	AY804032	AY790027	AY817189
Brycinus nurse	AY787970	AY791366	AY817302	AY804034	AY790029	AY817191
Phenacogrammus aurantiacus	AY788066	AY791428	AY817391	AY804111	AY790124	AY817270
Hepsetidae						
Hepsetus odoe	AY788030	AY791408	AY817356	AY804086	AY790089	AY817241
Citharinidae						
Citharinus citharus	AY787989	AY791378	AY817319	---	AY790048	---
Distichodontidae						
Distichodus sexfasciatus	AY788012	AY791396	AY817339	AY804071	AY790071	AY817226
Neolebias trilineatus	AY788063	AY791425	AY817388	AY804108	AY790121	AY817268
CYPRINIFORMES						
Cobitidae						
Misgurnus sp.	AY788053	---	AY817379	AY804103	AY790111	---
Cyprinidae						
Danio rerio	AY788011	---	AY817338	AY804070	AY790070	AY817225
Labeo sorex	AY788043	AY791415	AY817369	---	AY790101	AY817251
Gyrinocheilidae						
Gyrinocheilus sp.	AY788015	AY791399	---	AY804074	AY790074	AY817229
SILURIFORMES						
Callichthyidae						
Corydoras rabauti	NC004698	NC004698	---	---	---	---
Loricariidae						
Ancistrus sp.	AY787958	AY791354	AY817290	---	AY790016	AY817183
Bagridae						
Chrysichthys sp.	AY787957	AY791355	---	---	AY790017	AY817193
Heptapteridae						
Pimelodella sp.	AY787953	AY791351	AY817285	---	AY790011	AY817178
Ictaluridae						
Ictalurus punctatus	AY788040	AY791413	AY817366	---	AY790098	---

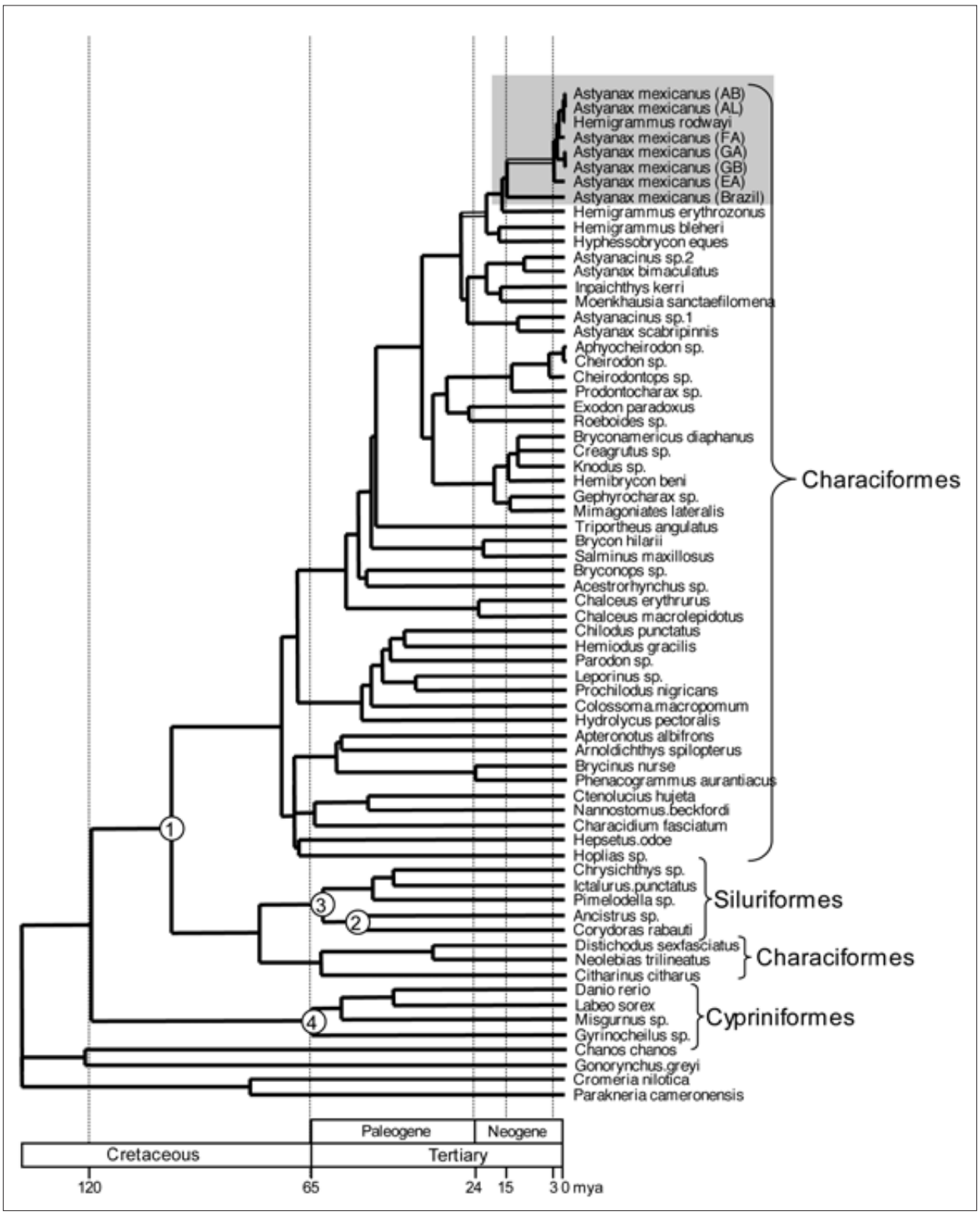

Fig. 1: Characiform divergence time chronogram estimated using a representative topology chosen from the set of 867 most parsimonious trees. White branches indicate branches where less than 75% of the most parsimonious trees were topologically congruent. The grey box indicates the clade of Astyanax mexicanus sequences. Fossil calibration nodes are numbered and correspond to Tab. 2. The major geologic periods are mapped onto the phylogeny.

Tab. 2: Taxonomy and ages of fossils used as calibrations for divergence time estimation. Node # refers to Fig. 1.

Taxonomy	Reference	Geologic age (MYA)	Node #
Otophysi			
Characiformes	Gayet, 1982	Late Cretaceous (65-99)	1
Cypriniformes			
Catostomidae	Cavender, 1986	Paleocene (54.8-65)	4
Siluriformes	Gayet & Meunier, 2003	late Campanian-early Maastrichtian (68.2-77.4)	3
Corydoras	Cockerell, 1925	Late Palaeocene (61-65)	2

RESULTS

Population-level divergence time estimations. Estimates of the mean divergence times were not significantly different between strict and relaxed clock and population growth models and calibration methods of the substitution rate, but confidence intervals under the fixed substitution rate approach were narrower, as expected. Hence only the time estimates under the strict clock model, constant population size and minimum and maximum mean substitution rates for both genes are provided. Comparing the cytb and ND2 estimates of divergence times for the *A. mexicanus* A and B lineages show several features. First, the estimated ranges of divergence for cave haplotypes within each lineage were similar between genes (cyt*b* and ND2) and lineages (A and B), placing the divergence among hypogean populations between 0.141-0.885 Ma for lineage A, and 0.084-0.575 Ma for lineage B (Tab. 3). When comparing the estimates among genes within a lineage, however, the divergence times of hypogean and epigean haplotypes are different, with cytb estimates providing generally older estimates.

Species-level divergence time estimation. Using the maximum parsimony ratchet, the selected Characidae, Characiform, and Otophysi sequences generated 867 trees of score 11758. The 50% majority rule consensus of these trees was similar to the published research that generated the data (Calcagnotto *et al.*, 2005). Because a fully resolved tree with branch lengths is required for AHRS divergence time estimation and because very few branches in the consensus tree collapsed (e.g. were in conflict), a random tree from the set of 867 was used (Fig. 1). The *A. mexicanus* sequences included in the analysis clustered with other Characidae species, although were not monophyletic with other Astyanax species *(A. bimaculatus* and *A. scabripinnis).* The divergence time estimates for the representative *A. mexicanus* cave fish populations generated using this phylogeny with Otophysi fossil calibrations agreed well with the estimates of hypogean haplotype divergence from cyt*b* and ND2 using substitution rates (Tab. 3). However, the estimates of cave versus surface population divergence times based on fossil calibrations were in better agreement with ND2 than with cytb estimates. This is particularly interesting, as the only gene included in this dataset for *A. mexicanus* was cyt*b*.

Tab. 3: Comparison of divergence time estimates using substitution rates and molecular clock methods for cytochrome b (cytb) and NADH dehydrogenase 2 (ND2) mitochondrial genes, and for molecular methods incorporating fossil dates as calibrations.

	Substitution Rates		Fossil Calibration
	Cytb	ND2	
	Min – Max (Ma)	Min – Max (Ma)	Min – Max (Ma)
Lineage A			
cave	0.261 – 0.885	0.141 - 0.331	0.2-0.3
cave vs. surface	0.588 - 2.00	0.256 – 0.599	0.4-0.5
Lineage B			
cave	0.169 – 0.575	0.084 - 0.196	0.1-0.1
cave vs. surface	1.524 – 5.181	0.877 – 2.055	1.7-2.2
Lineage A vs. Lineage B	1.741 – 5.922	1.053 – 2.472	1.7-2.2

DISCUSSION

Previous molecular studies of *A. mexicanus* phylogeography indicate that at least two independent invasions of surface Astyanax have occurred (Dowling *et al.,* 2002a; Strecker *et al.,* 2003, 2004). Our estimates of divergence time from two different methods and three different datasets are in general agreement about the divergence times among the cave haplotypes in each lineage (Tab. 3). These estimates place cave haplotype divergence times in the Pleistocene, when it is suggested that climatic cooling of surface waters led to the extinction of *Astyanax* in North America (Strecker *et al.,* 2004). In particular, our data show an interesting pattern for lineage B haplotypes, which are proposed to be the older of the two lineages. The recent divergence times estimated for lineage B haplotypes (0.084-0.575 Ma) supports the hypothesis that after the initial colonization event, subterranean routes of colonization were associated with fluctuating groundwater levels in the Pleistocene (Strecker *et al.,* 2004). The fact that estimated times of within lineage divergence were similar also suggests that the divergence of subterranean haplotypes in both lineages were influenced by the same processes.

In order to determine the evolutionary age of the subterranean lineage, and therefore estimate the time required for evolution of the troglomorphic form, the divergence of the hypogean haplotypes from epigean populations is needed. However, the estimates from our three datasets did not agree, with cyt*b* molecular clock methods estimating older divergence times than either ND2 or fossil calibrated estimates. Some of the discrepancy is due to the fact that different sets of surface populations were sampled in each study (Dowling *et al.,* 2002a; Strecker *et al.,* 2004). For example, the most closely related surface population in the cyt*b* study were from Belize (Strecker *et al.,* 2004) while there were no closely related surface populations to lineage B haplotypes in the ND2 study (Dowling *et al.,* 2002a). However, this makes the older cytb estimates even more notable because lineage B haplotypes have no evidence of introgressive hybridization with surface populations. If we consider just lineage B hypogean divergence from surface ancestors as an estimate of subterranean evolution, the estimated time for acquisition of the troglomorphic form is 0.877-2.055 Ma (Quaternary – Tertiary boundary) based on ND2 and fossil calibrations, while it is 1.524-5.181 Ma (Pliocene) based on cyt*b*.

Although the estimates of divergence times among the three different datasets did not agree, comparison of estimates between the lineages show that lineage A diverged from surface ancestors more recently than lineage B (Tab. 3). This more recent divergence from epigean populations is congruent with previous hypotheses, that either lineage A populations represent a more recent subterranean invasion, or that they are an older invasion masked by more recent mitochondrial introgressive hybridization with surface forms (Dowling *et al.,* 2002a). In the few studies that have looked at other markers (allozymes, microsatellites, and RAPDs), it has been suggested that at least Chica and Pachón populations are the result of surface introgression (Avise & Selander, 1972; Espinasa & Borowsky, 2001; Strecker *et al.,* 2003). Furthermore, based on the degree of variability in troglomorphic features of each lineage A population, it has been suggested that different populations represent different degrees and patterns of surface introgression. In order to more accurately determine both the patterns of introgression in the lineage A populations, as well as the underlying relationships of the cave populations to each other in order to estimate subterranean evolutionary times, studies investigating more types of markers are needed.

Previous research of *A. mexicanus* populations throughout Mexico (including cavefish lineages A and B) estimated haplotype divergences to range from 1.8 – 4.5 Ma (Strecker e*t al.,* 2004). Our estimates suggest that divergence times among cave haplotypes and between lineage A cave and epigean haplotypes are much younger than this; however, hypogean divergences from surface ancestors in lineage B are concordant with these older dates.

The evolutionary history of cave adaptation in *A. mexicanus* is complex. Based on mitochondrial molecular clock estimates, our estimates of divergence times are congruent with previous hypotheses by showing lineage B to be a phylogenetically older subterranean lineage, with more recent divergence among subterranean systems. However, this study also provides quantitative dates for these events. Lineage A populations are estimated to be younger; however, these dates only represent mitochondrial lineages. Several of the populations in lineage A have been shown to be introgressed with surface forms (Chica, Pachón, and Subterraneo). To our knowledge, the hypothesis of surface introgression has not been investigated in the remaining lineage A populations (Molino, Pichijumo, and Yerbaniz). Understanding the patterns of introgression in all of the lineage A populations, and estimating the actual subterranean evolutionary time, requires investigating additional nuclear markers.

CONCLUSIONS

Features including colonization routes (stream capture) and the existence of both epigean and cave-adapted hypogean populations make *A. mexicanus* an attractive system for investigating the subterranean evolutionary time necessary for acquisition of the troglomorphic form. If it is possible to estimate the divergence time of closely related cave versus surface populations, we can estimate the age of subterranean occupancy. This same divergence time also has relevancy to geologic processes in the karst system by providing a rough estimate of the age of subterranean stream capture in particular regions. Based on published sequence data, we have estimated divergence times for three events in the evolutionary history of troglomorphic *A. mexicanus* populations. First, divergence times among cave haplotypes in both lineages occurred in the Pleistocene, possibly correlating with fluctuating water levels allowing the colonization, and subsequent isolation of, new subterranean habitats. Second, in lineage A, *A. mexicanus* cave populations experienced introgressive hybridization events with surface populations recently. Finally, using divergence times of lineage B from surface populations as an estimate, the acquisition of the troglomorphic form in *A. mexicanus* is younger than 2.2 (fossil calibration) – 5.2 (cytb) Ma (Pliocene). Given that there are at least 30 caves known to contain populations of *A. mexicanus* (Espinasa *et al.,* 2001; Mitchell *et al.,* 1977), the number of independent invasions and instances of introgressive hybridization may be even higher than currently understood. In order to fully understand the number of independent invasions, the history of introgression with surface populations, and the divergence times of cave and surface populations, a broader survey of cave fish populations and of both nuclear and mitochondrial markers is needed.

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