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Molecular systematics of the Middle American genus *Hypopachus* (Anura: Microhylidae)

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ABSTRACT

We present the first phylogenetic study on the widespread Middle American microhylid frog genus *Hypopachus*. Partial sequences of mitochondrial (12S and 16S ribosomal RNA) and nuclear (rhodopsin) genes (1275 bp total) were analyzed from 43 samples of *Hypopachus*, three currently recognized species of *Gastrophryne*, and seven arthroleptid, brevicipitid and microhylid outgroup taxa. Maximum parsimony (PAUP), maximum likelihood (RAxML) and Bayesian inference (MrBayes) optimality criteria were used for phylogenetic analyses, and BEAST was used to estimate divergence dates of major clades. Population-level analyses were conducted with the programs NETWORK and Arlequin. Results confirm the placement of *Hypopachus* and *Gastrophryne* as sister taxa, but the latter genus was strongly supported as paraphyletic. The African phrynomerine genus *Phrynomantis* was recovered as the sister taxon to a monophyletic *Chiasmocleis*, rendering our well-supported clade of gastrophrynines paraphyletic. *Hypopachus barberi* was supported as a disjunctly distributed highland species, and we recovered a basal split in lowland populations of *Hypopachus variolosus* from the Pacific versant of Mexico and elsewhere in the Mesoamerican lowlands. Dating analyses from BEAST estimate speciation within the genus *Hypopachus* occurred in the late Miocene/early Pliocene for most clades. Previous studies have not found bioacoustic or morphological differences among these lowland clades, and our molecular data support the continued recognition of two species in the genus *Hypopachus*.

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1. Introduction

The microhylid genus *Hypopachus* (Keferstein, 1867), which currently includes two species in the lowlands and highlands of southeastern Texas, Mexico and Middle America, is a group of morphologically conservative, fossorial frogs. However, species of the genus *Hypopachus* vary considerably in coloration, intensity and distribution of pigmentation, and male advertisement calls (Nelson, 1973a, 1974). Unbeknownst to earlier workers, *Hypopachus* is sexually dimorphic in its pattern of foot webbing (EG, unpubl. data); this intraspecific and intersexual variation may have contributed to the current 23 available synonyms for the widespread and lowland species *Hypopachus variolosus* (Cope, 1866) and three for the Mesoamerican highland species *Hypopachus barberi* Schmidt, 1939 (Frost, 2011). The former species occurs from southern Texas, USA, to northern Costa Rica on the Atlantic versant, and from southern Sonora, Mexico, to central Costa Rica on the Pacific versant (McCranie and Wilson, 2002); its habitat ranges from desert flats to rainforests (Frost, 2011; Savage, 2002).

The genus *Hypopachus* currently includes two species that were extensively studied by Nelson (1973a, 1974), who examined adult morphology (with multivariate statistical analyses), color pattern, and mating calls from dozens of localities throughout the range. However, Savage (2002, p. 395) pointed out that, "[Nelson's] analysis left unresolved whether the nominal upland form, *H. barberi*, was really distinct from *H. variolosus*, since no diagnostic feature unequivocally separates these population systems." Some of the characters traditionally used to distinguish the highland and lowland species include size and distance between the inner and outer metatarsal tubercles (inner and outer metatarsal tubercles separated from each other by a multiple of the width of the outer metatarsal tubercle; Köhler et al., 2006); however, we have observed intra- and interspecific variation of this character, which suggests it is not a reliable diagnostic character for the species.

Compared to most other New World anurans, microhylids are relatively dull-colored, globular, rotund, and small. Many species are burrowers, with secretive habits and brief reproductive seasons – factors contributing to their rareness in collections. These characteristics might help explain why New World microhylids have

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received relatively little taxonomic and systematic attention from herpetologists (Duellman and Trueb, 1986). Following taxonomic treatments of New World microhylines (Carvalho, 1954; Parker, 1934), a subfamily of microhylids that formerly included taxa in the New World and Southeast Asia, Donnelly et al. (1990) and Wild (1995) used morphological data to construct phylogenies of the New World microhylids. Wild (1995) used novel morphological data and larval characters from Donnelly et al. (1990) to construct a phylogeny of all New World microhyline genera. However, Wild (1995) did not specify outgroups and assumed the monophyly of the group, which has been partially refuted by recent large-scale phylogenetic analyses (e.g., Frost et al., 2006; Van der Meijden et al., 2007). The foci of these and subsequent molecular studies (e.g., Van der Meijden et al., 2004, 2007; Frost et al., 2006; Van Bocxlaer et al., 2006), were at the level of family or higher taxonomic categories, and included a limited number of New World microhylids (only one included data from the genus *Hypopachus*). Van der Meijden et al. (2007) published a global-scale phylogeny of microhylid frogs that included *H. variolosus*, which was recovered as sister to *Gastrophryne carolinensis*, the type species of both genera. No molecular study to date has included *H. barberi*, or examined intraspecific variation within either species of *Hypopachus*.

Frost and Hillis (1990) predicted that most wide-ranging polytypic species of amphibians and reptiles eventually would be found to consist of several evolutionary lineages. Recent molecular studies have modified decades-old taxonomy of Middle American herpetofauna to include more species than recognized by morphology alone (e.g., Parra-Olea et al., 2004; Shaffer et al., 2004; Mendelson et al., 2005; Pfeiler and Markow, 2008; Zarza et al., 2008; Daza et al., 2009; Douglas et al., 2010). The chaotic taxonomic history (Frost, 2011) and widespread distribution of the Middle American microhylid genus *Hypopachus* render it an ideal taxon for testing species boundaries with molecular data.

2. Materials and methods

2.1. Taxon sampling

We sequenced eight samples of *H. barberi* and 35 samples of *H. variolosus* from across the entire known distribution of the species (Fig. 1). Based on previous studies of microhylid relationships (Frost et al., 2006; Van der Meijden et al., 2007), we included several outgroups in the study, including: three species of *Gastrophryne* (the sister group to *Hypopachus*), one species of *Elachistocleis*, three species of *Chiasmocleis*, one species of *Phrynomantis* (phrynomerine), and two arthroleptid species in the genera *Arthroleptis* and *Leptopelis*. *Breviceps mossambicus* was used to root phylogenetic trees (Loader et al., 2004; Van der Meijden et al., 2004).

2.2. Laboratory protocols

Frog tissue samples were collected as frozen whole tissue (muscle or liver), in ethanol or lysis buffer (0.5 M Tris/0.25% EDTA/2.5% SDS pH 8.2). We isolated genomic DNA using standard phenol–chloroform extractions (Hillis et al., 1996), or with DNeasy Tissue Kits (Qiagen, Valencia, CA). Although the phenol–chloroform method usually resulted in higher yields of DNA, the Qiagen kits often isolated DNA when the former method failed to do so. Double-stranded PCR amplifications were executed with 25 μl of Promega Master Mix (Promega, Madison, WI), 23 μl of purified water, 0.4 μl of forward and reverse primers, and approximately 0.5 μl of DNA (depending on strength of DNA isolation). A segment of about 400 base pairs (bp) from the 12S rRNA gene was amplified with primers 12Sa 5′-AAACTGGGATTAGATACCCCACTAT-3′ and 12Sb 5′-GAGGGTGACGGGCGCTGTGT-3′ with the following sequencing conditions: 94 °C for 2.5 min, 53 °C for 1 min, and 72 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1.5 min. A segment of about 600 bp from the 16S rRNA gene was amplified with primers 16SH10 5′-TGATTACGC-TACCTTTGCACGGT-3′ and 16SL2A 5′-CCAAACGAGCCTAGTGA-TAGCTGGTT-3′ with the following sequencing conditions: 94 °C for 2 min, 50 °C for 1 min, and 72 °C for 1.5 min, followed by 34 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1.5 min. A segment of about 310 bp from the rhodopsin nuclear gene was amplified with primers Rhod 1A/D (Hoegg et al., 2004) with the following conditions: 94 °C for 2 min, 49 °C for 1 min, and 72 °C for 1 min, followed by 34 cycles of 94 °C for 1 min, 49 °C for 1 min, 72 °C for 1 min, and one final cycle of 72 °C for 6 min.

Products from PCR were purified with AMPure magnetic beads (Agencourt Bioscience, Beverly, MA) or the GenElute DNA Purification Kit (Sigma–Aldrich, St. Louis, MO). Purified products were cycle-sequenced with the dideoxy chain termination method with the SequiTherm Excel II DNA sequencing kit (Epicenter Technologies, Omaha, NE) or BigDye® Terminator Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA). SequiTherm sequencing products were run on 4% acrylamide (44-cm and 66-cm plates) gels with a Licor DNA 4000L or 4300 automatic sequencer. BigDye sequencing products were run on an ABI 3130xl automated sequencer at the DNA Core Facility at the University of Texas at El Paso (UTEP). Forward and reverse sequence contigs for each sample were assembled and edited in BaseImagr, AlignIR (Licor Biotechnology) or Seqman (DNAStar) for visual inspection of data for mismatches of aligned positions to confirm or manually correct automatic readings. All sequences generated in this study were deposited in GenBank (Table 1), and the final combined alignment is available on Treebase (http://purl.org/phylo/treebase/phylows/study/TB2:S11472).

2.3. Sequence alignment, phylogenetic analyses and divergence time estimates

An initial alignment of each gene was produced in MEGALIGN (DNA Star) with the Clustal W algorithm, and manual adjustments were made in MacClade 4.08 (Maddison and Maddison, 2005). Rhodopsin data were translated to amino acids in MacClade to confirm conservation of the amino acid reading frame, ensure proper alignment, and check for premature stop codons. After preliminary phylogenetic analyses of data from each gene resulted in congruent topologies, all data were combined for subsequent analyses.

Phylogenetic relationships among the samples were assessed with maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) optimality criteria in the programs PAUP* 4.0b10 (Swofford, 2002), RAxML (Stamatakis, 2006), and MrBayes 3.1 (Ronquist and Huelsenbeck, 2003), respectively. For MP analyses, the heuristic search algorithm was used with 25 random-addition replicates, accelerated character transformation, and tree bisection-reconnection branch swapping, zero-length branches were collapsed to polytomies, and indels were treated as missing data. We used non-parametric bootstraps (1000 pseudoreplicates) to assess node support in resulting topologies (Felsenstein, 1985). Comparisons of uncorrected *p* sequence mitochondrial divergence data were made with the complete deletion option in MEGA4 (Tamura et al., 2007).

The corrected Akaike Information Criterion in jModelTest (Posada, 2008) was used to find the model of evolution that best fit the data for subsequent partitioned ML and BI analyses. Following previous studies that demonstrated the importance of gene and codon partitions for accurate BI analyses (e.g., Castoe et al., 2004;

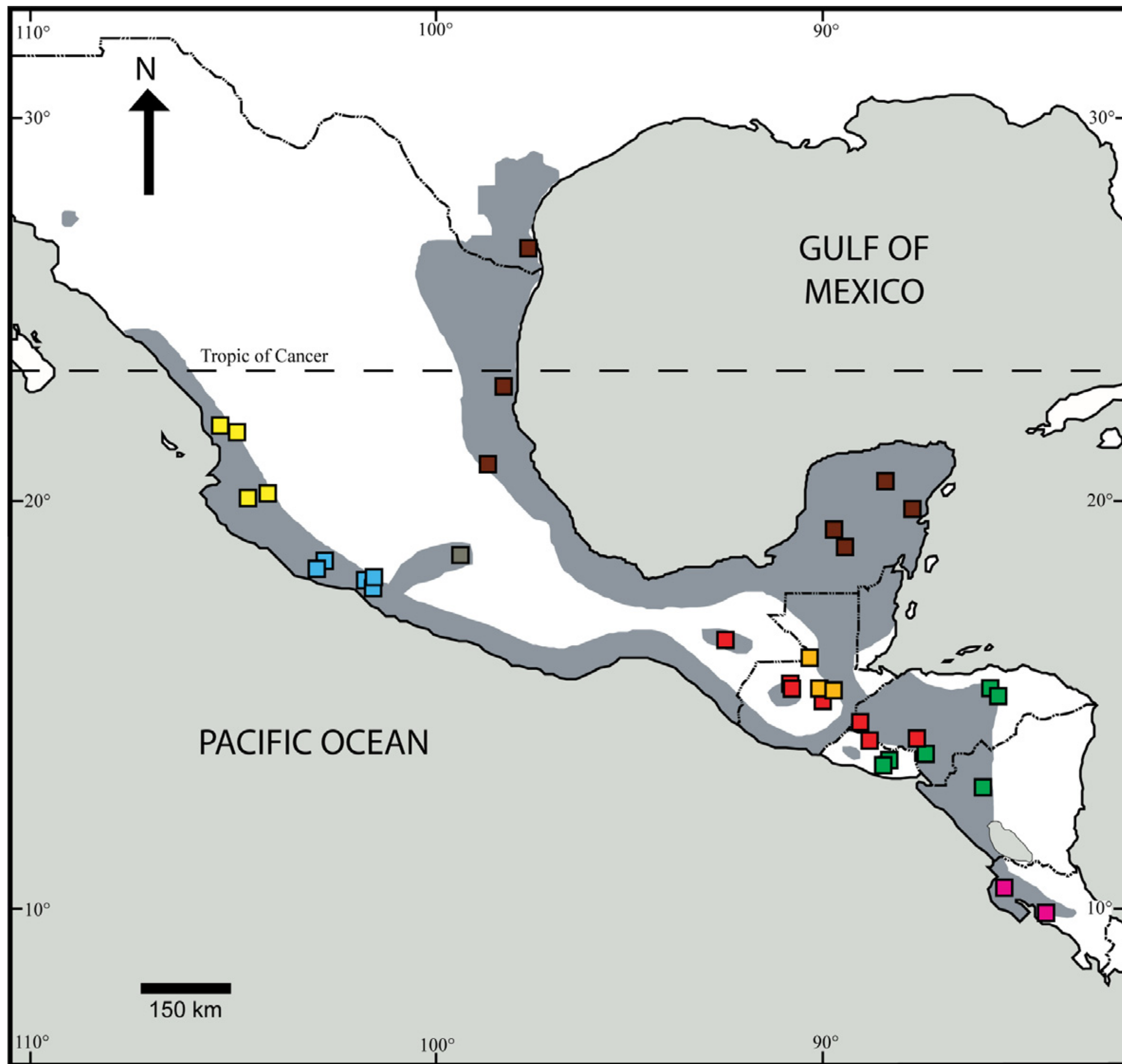

Fig. 1. Map of Mesoamerica showing the known distribution of *Hypopachus* in gray shading, and the locations of samples used in this study. Colors of samples correspond to lineages shown in Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Brandley et al., 2005), we estimated models for 12S, 16S, and each codon position of rhodopsin. RAxML analyses were executed with partitioned datasets (one each for 12S and 16S, and one for each codon position of rhodopsin using the least restrictive model) and 100 replicate ML inferences were performed for each analysis. Each analysis was initiated with a random starting tree, and employed the rapid hill-climbing algorithm (Stamatakis et al., 2007). Clade support was assessed with 1000 bootstrap replicates, also with the rapid-hill climbing algorithm (Stamatakis et al., 2008).

Partitioned Bayesian analyses were conducted with default priors. Analyses were initiated with random starting trees and run for 20,000,000 generations; Markov chains were sampled every 1000 generations. Convergence was checked by importing the trace files (p files) from the MrBayes output to the computer program Tracer v1.3 (http://tree.bio.ed.ac.uk/software/tracer/), which plots the likelihood values against generation number. Once the graphical plot leveled off, convergence had been met; we conservatively discarded 25% of trees as "burn in." Four separate analyses with two independent chains were executed to check for convergence of log-likelihoods in stationarity (Huelsenbeck and Ronquist, 2001; Leaché and Reeder, 2002). To test the monophyly of clades recovered in our phylogenetic analyses, we used the Shimodaira–Hasegawa (SH) and approximately unbiased (AU) tests as implemented in CONSEL V0.1i (Shimodaira and Hasegawa, 2001; Shimodaira, 2002).

Estimates of divergence times for lineages identified in the phylogenetic analyses were calculated under a Bayesian statistical framework with the program BEAST version 1.6.1 (Drummond and Rambaut, 2007). For a calibration point, we used a fossil of *Gastrophryne* cf. *carolinensis* described by Holman (1965) from the Hemingfordian North American Stage of the early Miocene (ca. 18 mya) in Florida, which was used as the minimum age for the genus *Gastrophryne*. To ensure BEAST analyses reached effective sample size (ESS) values >200 for the posterior and prior values, we used unlinked substitution and clock models for the separate mitochondrial and nuclear datasets, GTR substitution and gamma site heterogeneity models for each dataset, relaxed clock (uncorrelated lognormal) and Yule process speciation tree models, and an exponential prior on the *Gastrophryne* fossil calibration (Ho, 2007). Each MCMC chain was run for 20,000,000 generations (sampled every 1000 generations) to ensure ESS values were higher than 200. BEAST runs were repeated four times to ensure the stability and convergence of the MCMC chains. Combined results from the four analyses were analyzed with the program Tracer version 1.5 (Rambaut and Drummond, 2007).

Table 1
Anuran specimens sampled for molecular data, and their associated voucher numbers and locality information. Museum abbreviations are listed in Leviton et al. (1985), except for Museo de Zoología, Facultad de Ciencias, Universidad Autonoma de Mexico (MZFC). Uncatalogued specimen abbreviations include: Rafael O. de Sá field series (RDS) and Brice P. Noonan field series (BPN).

Sample	Locality	Voucher No.	GenBank Accession Numbers		
			12S	16S	Rhodopsin
Breviceps mossambicus	Tanzania: Morogoro	RDS 903	JF836942	JF836996	JF837044
Arthroleptis adolfifriederici	Democratic Republic of the Congo: South Kivu: Kahuzi-Biega National Park, Mugaba, 2289 m (S02.27288°, E28.66208°)	UTEP 20311	JF836941	JF836995	JF837043
Leptopelis kivuensis	Democratic Republic of the Congo: South Kivu: Kahuzi-Biega National Park, Mugaba, 2289 m (S02.27288°, E28.66208°)	UTEP 20137	JF836940	JF836994	JF837042
Phrynomantis microps	Ghana: Eastern Region: Muni Lagoon	KU 290437	JF836939	JF836993	JF837041
Chiasmocleis bassleri	Peru: Loreto: 1.5 km N Teniente Lopez	KU 222104	JF836936	JF836990	JF837038
C. hudsoni	Suriname (5.18765; −55.6521°)	BPN 925	JF836937	JF836991	JF837039
C. ventrimaculata	Peru: Madre de Dios: Cuzco Amazonico, 15 km E Puerto Maldonado	KU 215540	JF836938	JF836992	JF837040
Elachistocleis bicolor	Paraguay: Parque Nacional San Luis de la Sierra (22°40′S; 57°21′W)	KU 289149	JF836935	JF836989	JF837037
Gastrophryne carolinensis 1	USA: Louisiana: Ouachita: Russell Sage Wildlife Reserve	KU 289624	JF836899	JF836953	JF837006
G. carolinensis 2	USA: Alabama: Washington Co: Boykin Wildlife Management Area	UTEP 19907	JF836901	JF836955	JF837007
G. carolinensis 3	USA: Florida: Putnam Co: Caravelle Ranch Wildlife Management Area	UTEP 20087	JF836900	JF836954	–
Gastrophryne olivacea 1	USA: Texas: Travis Co: Austin	UTEP 18274	JF836902	JF836956	–
G. olivacea 2	USA: Texas: Hudspeth Co: Indio Mountains Research Station	UTEP 19815	JF836903	JF836957	JF837008
G. usta	Mexico: Guerrero: Soyatepec, 17.32791 N 99.55802 W, 738 m	UTA A-60366	JF836898	JF836952	JF837005
Hypopachus barberi 1	Mexico: Chiapas: 29.7 km NE Huixtla by Motozintla de Mendoza Rd., forested slope above road summit; 2011 m	MVZ 160939	JF836886	JF836943	JF836997
H. barberi 2	Guatemala: Baja Verapaz: Carretera Salama-Pantin, 1580 m	UTA A-57153	JF836888	JF836945	–
H. barberi 3	Guatemala: El Quiché: Uspantán, El Chimel	UTA A-50963	JF836887	JF836944	JF836998
H. barberi 4	El Salvador: Chalatenango: Cerro El Pital, 2300 m	KU 291248	JF836889	JF836946	JF836999
H. barberi 5	Guatemala: El Quiché: Uspantan (within city limits), 15°15.93′N, 90°52.07′W, 1825 m	UTA A-55222	JF836922	JF836976	JF837025
H. barberi 6	Honduras: La Paz: cattle pond ca. 6 km N of Guajiquiro, 2160 m (14.146537°, −87.844239°)	UF 161686	JF836891	–	JF837000
H. barberi 7	Honduras: La Paz: cattle pond ca. 6 km N of Guajiquiro, 2160 m (14.146537°, −87.844239°)	UF 161687	JF836892	–	JF837001
H. barberi 8	Honduras: Ocotepeque: Reserva Biologica El Guisayote, 2230 m (14.438562°, −89.064444°)	UF 161688	JF836890	–	–
H. variolosus 1	USA: Texas: southeastern Texas	RDS 277	JF836893	JF836947	–
H. variolosus 2	Mexico: Nayarit: Huajimic	MZFC 13101	JF836896	JF836950	–
H. variolosus 3	Mexico: Jalisco: Sta Maria de Los Angeles-Bolanos: Rio Cartagena, 21.98340 N, 103.34106 W, 1602 m	UTA A-57702	JF836909	JF836963	JF837014
H. variolosus 4	Mexico: Jalisco: La Mascota: 20.53515 N, 104.80064 W, 1133 m	UTA A-57706	JF836910	JF836964	–
H. variolosus 5	Mexico: Nayarit: Navarrete, 21.646940°, −105.115280° (max error distance: 0.952 km; datum: NAD27)	MVZ 144018	JF836907	JF836961	JF837012
H. variolosus 6	Mexico: Nayarit: Navarrete, 21.646940°; −105.115280° (max error distance: 0.952 km; datum: NAD27)	MVZ 144019	JF836908	JF836962	JF837013
H. variolosus 7	Mexico: Michoacán: AOR Mexican Hwy 120 between Apatzingan and Tepalcatepec, 19.15663°N, 102.47886°W, 370 m	UTA A-56885	JF836913	JF836967	JF837017
H. variolosus 8	Mexico: Michoacán: AOR between Tepalcatepec and Coalcoman, 18.79473°N, 103.14625°W, 1030 m	MZFC 17650	JF836914	JF836968	JF837018
H. variolosus 9	Mexico: Guerrero: San Vicente de Benites, 17.29061°N, 100.27955°W, 951 m	UTA A-56888	JF836916	JF836970	–
H. variolosus 10	Mexico: Guerrero: San Vicente de Benites, 17.29061°N, 100.27955°W, 951 m	MZFC 17641	JF836917	JF836971	JF837020
H. variolosus 11	Mexico: Guerrero: Carretera Rio Santiaga-San Vicente, 17.30069°N, 100.27911°W, 904 m	MZFC 17642	JF836915	JF836969	JF837019
H. variolosus 12	Mexico: Yucatán: Cenote Chamac-Kinchil	MZFC uncatalogued (S/D 00000 field No.)	JF836897	JF836951	JF837004
H. variolosus 13	Mexico: Campeche: Zona Arqueológica de Calakmul, 18°06′26″N, 89°48′34″W	No voucher (RDS tissue collection)	JF836918	JF836972	JF837021
H. variolosus 14	Mexico: Tamaulipas: Hacienda Acuna, 23.20452°N, 98.43519°W, 860 m	UTA A-56889	JF836911	JF836965	JF837015
H. variolosus 15	Mexico: Querétaro: Carretera El Lobo San Juan Del Rio Hwy 120, 21.25670°N, 99.25301°W, 1111 m	UTA A-56886	JF836912	JF836966	JF837016
H. variolosus 16	Mexico: Quintana Roo: no specific locality	MZFC uncatalogued (CA 560 field No.)	JF836895	JF836949	JF837003
H. variolosus 17	Mexico: Campeche: Carretera Xpujil-Bel-Ha, 18.89567°, −89.35042°, 197 m	UTA A-60454	JF836919	JF836973	JF837022
H. variolosus 18	Mexico: Campeche: Hwy. 186, 22.4 mi E (by road) Escárcega, 18.601389°; −90.421389° (max error distance: 2.458 mi; datum: NAD27)	MVZ 164760	JF836920	JF836974	JF837023
H. variolosus 19	Guatemala: Petén: Aguada near Hotel Villa Maya: 1.9 km N of Santa-Elena – El Remate Hwy on road to Peténchel	UTA A-53261	JF836925	JF836979	JF837028
H. variolosus 20	Guatemala: Baja Verapaz: Salama, Salama Hospital Field	UTA A-50968	JF836921	JF836975	JF837024
H. variolosus 21	Guatemala: Zacapa: Cabañas, Aldea El Arenal	UTA A-50971	JF836923	JF836977	JF837026
H. variolosus 22	Guatemala: Zacapa: Cabañas, Aldea El Arenal	UTA A-50973	JF836924	JF836978	JF837027
H. variolosus 23	Honduras: Olancho: San Estebán, Aldea Las Trojas	UTA A-50667	JF836926	JF836980	JF837029
H. variolosus 24	Honduras: Olancho: Quebrada San Lorenzo ca. 3 km S of San Lorenzo Ariba, 220 m	UF 14244	JF836928	JF836982	JF837030
H. variolosus 25	Honduras: Francisco Morazán: Tegucigalpa, 14.051°N, 87.219°W, 1000 m	UTA A-56790	JF836927	JF836981	–

H. variolosus 26	El Salvador: San Miguel: Volcán de San Miguel, 740 m	KU 291269	JF836930	JF836984	JF837032
H. variolosus 27	El Salvador: Usulután: Cerro del Tigre, 13°28.37′N, 88°26.21′W, 1100 m	KU 289842	JF836929	JF836983	JF837031
H. variolosus 28	El Salvador: San Miguel: Volcán de San Miguel, 740 m	KU 291270	JF836931	JF836985	JF837033
H. variolosus 29	Nicaragua: Matagalpa: Comarca Peñas Blancas, Finca San Sebastián	UTA A-51790	JF836932	JF836986	JF837034
H. variolosus 30	Costa Rica: Puntarenas: Reserva Biologia Carara; 1.9 rd mi S of Tarcoles River on Hwy 34	TCWC 83293	JF836934	JF836988	JF837036
H. variolosus 31	USA: Texas: Willacy County: N of La Sal Vieja on Rowland Co. Rd, 4.7 miles	UTA uncatalogued (RM 685 field No.)	JF836894	JF836948	JF837002
H. variolosus 32	Costa Rica: Guanacaste: Cafetal, Sector Santa Rosa	UCR 17777	JF836933	JF836987	JF837035
H. variolosus 33	Mexico: Morelos: Mpio. Tepoztlán: San Andrés de la Cal,18.96135 N, 99.10912 W, 1462 m	UTA A-60368	JF836904	JF836958	JF837009
H. variolosus 34	Mexico: Morelos: Mpio. Tepoztlán: San Andrés de la Cal,18.96135 N, 99.10912 W, 1462 m	UTA A-60457	JF836906	JF836960	JF837011
H. variolosus 35	Mexico: Guerrero: Soyatepec-El Ocotito, 17.30623°N, 99.56733°W, 715 m	UTA A-60367	JF836905	JF836959	JF837010

2.4. Haplotype networks and analyses of geographic and population structuring

We used NETWORK 4.600 (http://www.fluxus-engineering.com) to construct haplotype median-joining networks (Bandelt et al., 1999). Networks were constructed from the combined 12S and 16S dataset for all samples of *Hypopachus* (weights = 10 and $\varepsilon = 0$); nuclear data had minimal variation and were not included. We computed genetic diversity (nucleotide and haplotype diversity, and mean number of pairwise differences) within the different groups identified from the phylogenetic analyses with the program ARLEQUIN 3.5 (Excoffier and Lischer, 2010). This program was also used to conduct a hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992) to assess the most probable configuration and geographic subdivision. Populations were grouped into two, three, four, five and six groups according to different geographic hierarchies that matched the lineages recovered in our phylogenetic analyses. Groupings that maximized values of among group variation (F_{CT}) and were statistically significant indicated the most parsimonious geographic subdivisions (Zhang et al., 2010). Finally, ARLEQUIN was used to estimate mismatch distributions for several widespread clades to test for sudden population expansion (Patou et al., 2010). Assuming the infinite sites model, the mismatch distribution is smooth and often unimodal as a result of population expansion, whereas for stationary populations the distribution is ragged and often multimodal (Harpending, 1994; Harpending et al., 1998; Rogers and Harpending, 1992). We also implemented Fu's *F*s and Tajima's *D* neutrality tests as additional assessments of population expansion (Fu, 1997; Zhang et al., 2010).

3. Results

3.1. Alignment, phylogenetic analyses and haplotype networks

The total alignment for the dataset included 1275 bp (12S = 394 bp; 16S = 583 bp; rhodopsin = 298 bp). Alignment of all datasets did not result in any ambiguous regions and no stop codons were detected in the rhodopsin dataset; 417 characters were parsimony-informative (32.7%) sites. Three samples of *H. barberi* from Honduras failed to amplify for 16S (these were excluded from NETWORK and Arlequin analyses), and two samples of *Gastrophryne* and seven samples of *Hypopachus* failed to amplify for rhodopsin (Table 1).

The following models of nucleotide substitution were selected by jModeltest for ML and BI analyses: 12S (GTR + I + Γ); 16S (TiM3 + Γ); rhodopsin 1st codon position (TPM2uf), rhodopsin 2nd codon position (F81), rhodopsin 3rd codon position (TPM2uf + Γ). If a given model was not an option in RAxML or MrBayes, the least restrictive available model (i.e., GTR) was used. The topology of trees generated from MP, ML, and BI analyses were nearly identical, with the only difference related to the position of *Gastrophryne usta*, which was weakly supported (50% bootstrap support) as sister to the *G. carolinensis*/*G. olivacea* clade in the MP analysis (Fig. 2). The MP analysis of the dataset resulted in 3882 most parsimonious trees (length = 1757, CI = 0.538, RI = 0.694); the likelihood score of the most optimal tree in the ML analysis was −9737.457540.

Strongly supported clades (>70% bootstrap and 0.95 posterior probability [pp]) from all three analyses (Fig. 2A) included: a monophyletic *Hypopachus;* a monophyletic *H. barberi* (from highland localities in Chiapas [Mexico], Guatemala, Honduras and El Salvador); and a monophyletic *H. variolosus*. Within *H. variolosus*, there was a well-supported, basal split between the Nayarit, Morelos, and Guerrero clades from the Pacific versant of Mexico (WMX), and the Texas, Central, Costa and Baja clades from elsewhere in the

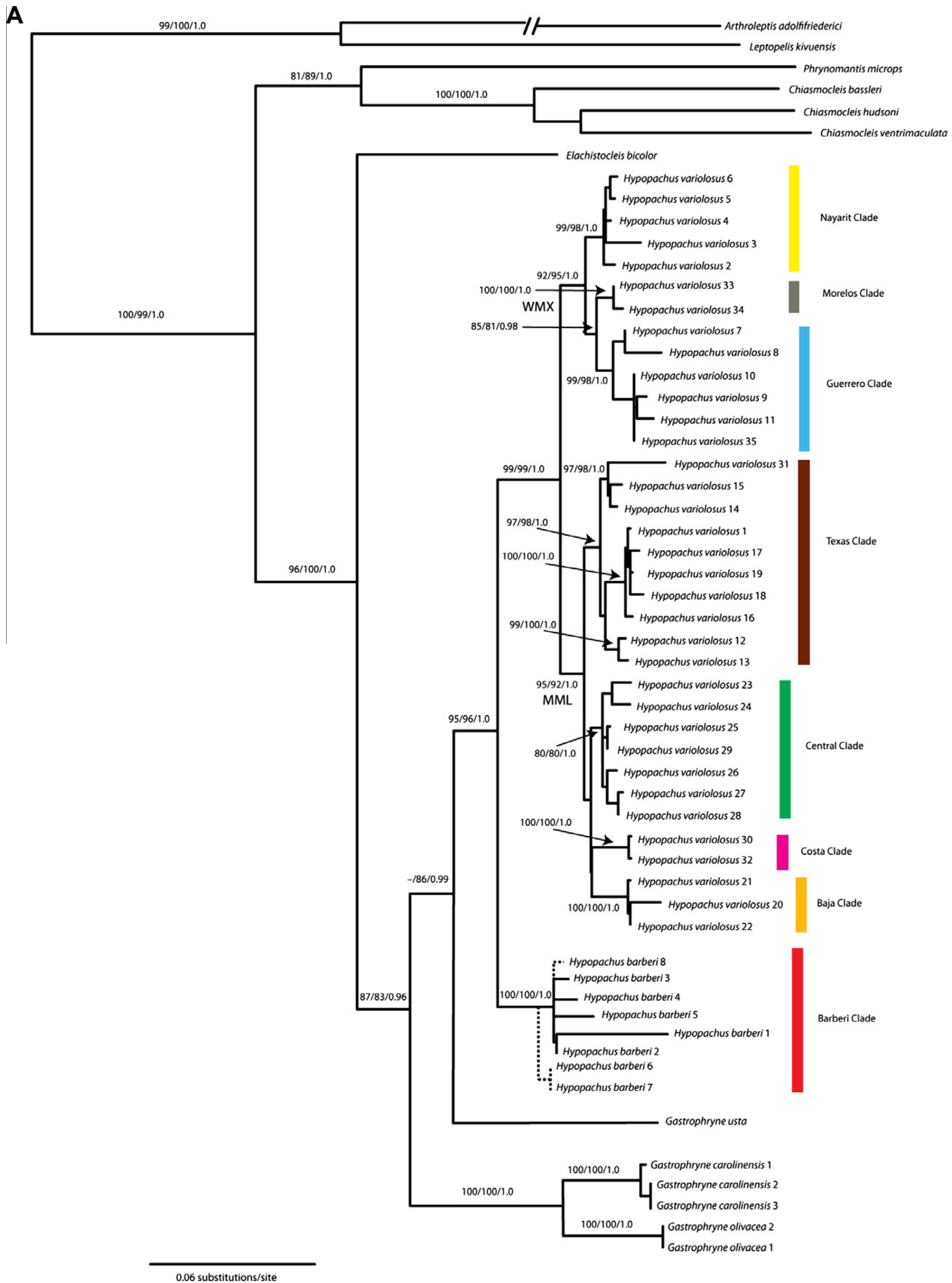

Fig. 2. Preferred maximum likelihood phylogeny (A) generated with RAxML using combined 12S, 16S and rhodopsin datasets. Phylogenetic tree was rooted with the outgroup taxon *Breviceps mossambicus* (not shown). The phylogenetic position of three samples of *Hypopachus barberi* were inferred from 12S and rhodopsin data only, and are shown with a dashed line. Branch support values are maximum parsimony/maximum likelihood/Bayesian inference. Haplotype network (B) obtained from the analysis of the mtDNA dataset (12S and 16S) are labeled according to their locations and sample ID. Haplotype connecting lines represent single mutations unless indicated otherwise (in parentheses). Median vectors introduced by the NETWORK algorithm are shown as small black circles. Hb = *Hypopachus barberi*; Hv = *Hypopachus variolosus*.

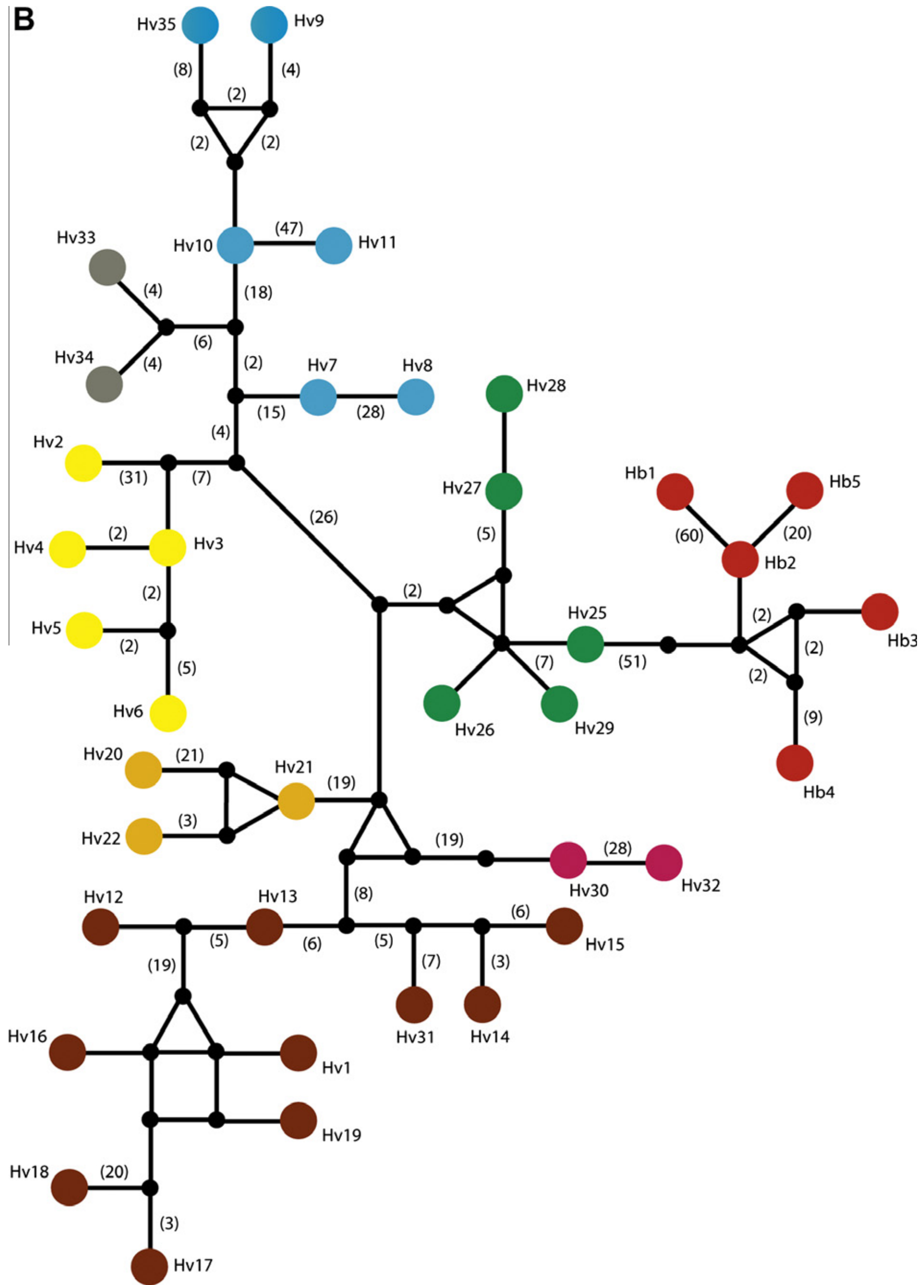

Fig. 2 (*continued*)

Mesoamerican lowlands (MML). The corresponding haplotype network (Fig. 2B) also showed two groups corresponding to the WMX and MML clades, differing by 26 mutations. Samples of *H. barberi* were separated into a third group from the Central Clade by 51 mutations (Fig. 2B). Maximum likelihood and BI analyses strongly supported *Gastrophryne usta* as sister to *Hypopachus*, and all analyses recovered moderate support for the position of a *G. carolinensis* + *G. olivacea* clade as sister to the *G. usta*/*Hypopachus* clade. Our analyses strongly supported *Elachistocleis* as sister to the *Gastrophyrne*/*Hypopachus* clade, a monophyletic *Chiasmocleis*, and a paraphyletic Gastroprhyninae because of the well-supported inclusion of the African phrynomerine genus *Phrynomantis* (moderately supported as sister to *Chiasmocleis*). Divergence times estimated from BEAST are listed in Table 2. The earliest lineage divergence within the genus *Hypopachus* occurred in the late

Table 2
Results of estimated divergence times (in millions of years) from BEAST analyses. Node names correspond to clades shown in Fig. 1.

Node	Mean	Standard error of mean	95% HPD lower	95% HPD upper	Effective sample size
Gastrophryne	18.83	0.03	18.00	20.49	67904.26
Hypopachus	11.71	0.08	7.37	15.98	859.96
WMX	6.00	0.05	3.00	9.38	1044.86
MML	6.38	0.06	3.38	9.60	831.88
Barberi	4.29	0.07	1.48	7.84	677.31
Nayarit	2.95	0.04	0.76	5.58	985.47
Morelos	0.86	0.01	0.03	2.26	4049.04
Guerrero	2.95	0.03	1.09	5.10	1345.57
Texas	4.30	0.05	1.96	6.94	843.01
Central	3.26	0.04	1.23	5.63	1195.36
Costa	0.72	0.01	0.02	2.08	3085.51
Baja	1.50	0.02	0.25	3.26	1289.07

Miocene, with subsequent divergences occurring in the Pliocene and Pleistocene.

3.2. Sequence variation and monophyly tests

The largest uncorrected *p* genetic distance in the mitochondrial dataset was between the outgroups *Arthroleptis adolfifriederici* and *Breviceps mossambicus* (29.4%). Among samples of *Gastrophryne*, the largest uncorrected *p* mitochondrial genetic distance was between *G. usta* and *G. olivacea* (14.3%); divergences between *G. usta* and *G. carolinensis* ranged from 13.9–14.2%, but divergences between sister taxa *G. carolinensis* and *G. olivacea* ranged from 6.9–7.1%. Variation within well-supported clades of *Hypopachus* ranged from 0.2–5.2% for *H. barberi* (maximum variation only 1.7% when the divergent Chiapas sample was excluded), whereas the divergences for *H. variolusus* ranged from 0.0–3.6% for WMX and 0.0–4.0% for MML. Uncorrected *p* mitochondrial divergences between well-supported clades of *Hypopachus* were: 4.7–10.0% (*H. barberi* vs. MML), 5.7–10.4% (*H. barberi* vs. WMX), and 3.5–6.5% (MML vs. WMX). We tested the monophyly of two taxa that were recovered as paraphyletic in our analyses, *Gastrophryne* and Gastrophryninae. The monophyly of *Gastrophryne* was not rejected by the AU ($P = 0.090$) and SH ($P = 0.118$) tests, but the monophyly of Gastrophryninae was rejected by the AU ($P = 0.037$) test, and a near-significant result for the SH ($P = 0.00051$) test.

3.3. Population and geographic structure

All 40 included samples of *Hypopachus* had unique haplotypes. Genetic diversity estimates and neutrality tests within populations of *Hypopachus* are shown in Table 3; populations with low sample sizes included the Morelos, Costa and Baja groups ($n \leqslant 3$). Among all individuals, $\pi = 0.0489$, $h = 0.999$ and $\kappa = 46.13$, suggesting that haplotype diversity is high and nucleotide diversity is relatively low. This pattern suggests that modern populations have very low levels of gene flow among them, and it is likely that these populations evolved in relative isolation from each other. In the AMOVA (Table 4), the highest amount of genetic variance among groups ($F_{CT} = 0.406$, $P < 0.01$) was found with three groups ([Barberi] [Morelos, Nayarit, Guerrero] [Baja, Costa, Central, Texas]), which are congruent with the three well-supported clades for *H. barberi*, MML and WMX recovered in the phylogenetic analyses (Fig. 2).

Mismatch distributions were generated for geographically widespread groups with relatively large sample sizes, including the Barberi, Central, Guerrero and Texas clades (Fig. 3). All four clades showed multimodal patterns (Barberi raggedness index $r = 0.2$, $P = 0.51$; Central $r = 0.12$, $P = 0.63$; Guerrero $r = 0.13$, $P = 0.56$; Texas $r = 0.06$, $P = 0.33$), indicating stable or slowly declining populations and long-term demographic stability (Rogers and Harpending, 1992). Non-significant Fu's *Fs* tests (Table 3) for these clades support sequence evolution consistent with the expectation of selective neutrality and stable demographic history for the clades.

4. Discussion

4.1. Phylogeny and taxonomy of Hypopachus

Our results provide the first comprehensive analysis of *Hypopachus* relationships based on molecular data. The well-supported clades identified in this study are geographically structured and correspond to previously recognized species of *Hypopachus* (Frost, 2011; Nelson, 1973a, 1974). We confirm the recognition of disjunct highland populations as *H. barberi*, which is consistent with current taxonomy. The well-supported basal split among lowland samples of *Hypopachus variolosus* from western Mexico (WMX) and else-

Table 3
Genetic diversity estimates and tests of neutrality within *Hypopachus*, partitioned into genetic groups identified from the phylogenetic analyses in Fig. 2. π = nucleotide diversity; h = haplotype diversity; κ = mean number of pairwise differences; n = sample size.

Clade	n	π	h	κ	Fu's *Fs*	*P*-value	Tajima's *D*	*P*-value
Barberi	5	0.0339	1.0	31.20	1.027	0.433	−0.911	0.201
Nayarit	5	0.0122	1.0	11.60	−0.144	0.277	−0.654	0.357
Morelos	2	0.0032	1.0	3.00	1.098	0.442	0	1.0
Guerrero	6	0.0274	1.0	25.67	0.318	0.344	−0.185	0.452
Texas	10	0.0172	1.0	16.29	−2.24	0.079	0.202	0.627
Central	7	0.0103	0.952	9.81	0.117	0.428	−0.537	0.336
Costa	2	0.0011	1.0	1.00	0	0.25	0	1.0
Baja	3	0.0109	1.0	10.33	1.174	0.466	11946792.089	1.0
TOTAL	40	0.0489	0.999	46.13	−8.416	0.011	−0.4608	0.368

Table 4
Hierarchical analysis of AMOVA of *Hypopachus* populations.

Groups	F_{ST}	F_{SC}	F_{CT}	% Among groups	% Within populations
All populations	0.687***	–	–	68.68	31.32
2 Groups: [Barberi, Baja, Costa, Central, Texas] [Morelos, Nayarit, Guerrero]	0.719***	0.643***	0.212*	21.24	28.14
2 Groups: [Barberi, Morelos, Nayarit, Guerrero] [Baja, Costa, Central, Texas]	0.712***	0.642***	0.195*	19.50	28.81
2 Groups: [Barberi] [Baja, Costa, Central, Texas, Morelos, Nayarit, Guerrero]	0.773***	0.639***	0.372	37.16	22.71
3 Groups: [Barberi] [Morelos, Nayarit, Guerrero] [Baja, Costa, Central, Texas]	0.727***	0.541***	0.406**	40.57	27.31
3 Groups: [Barberi, Baja, Costa, Central, Texas] [Nayarit] [Morelos, Guerrero]	0.706***	0.656***	0.145	14.49	29.42
4 Groups: [Barberi] [Morelos, Guerrero] [Nayarit] [Baja, Costa, Central, Texas]	0.719***	0.533***	0.398**	39.80	28.12
4 Groups: [Barberi] [Baja, Costa] [Morelos, Guerrero, Nayarit] [Central, Texas]	0.709***	0.531***	0.381**	38.07	29.06
5 Groups: [Barberi] [Morelos, Guerrero] [Nayarit] [Central, Texas] [Baja, Costa]	0.703***	0.516***	0.385**	38.47	29.75
5 Groups: [Barberi] [Morelos, Guerrero, Nayarit] [Central, Texas] [Baja] [Costa]	0.709***	0.518***	0.397**	39.69	29.07
5 Groups: [Barberi] [Morelos, Guerrero, Nayarit] [Central] [Texas] [Baja, Costa]	0.695***	0.555***	0.316*	31.55	30.47
6 Groups: [Barberi] [Morelos, Guerrero, Nayarit] [Central] [Texas] [Baja] [Costa]	0.695***	0.538***	0.339*	33.93	30.50

* $P<0.05$.
** $P<0.01$.
*** $P<0.001$.

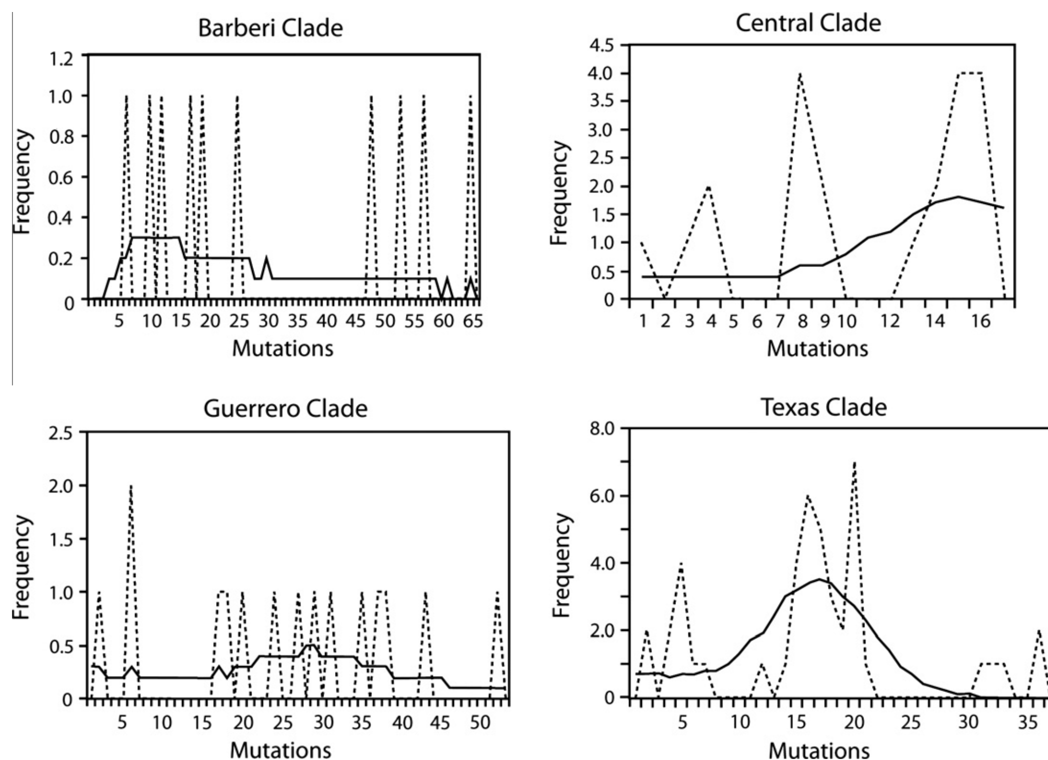

Fig. 3. Mismatch distributions computed for the combined 12S and 16S mitochondrial dataset for the Barberi, Central, Guerrero and Texas clades. Dashed line, observed distribution of pairwise differences; solid line, expected distribution in the case of sudden demographic expansion.

where in Mesoamerica (MML), and the uncorrected *p* mitochondrial sequence divergence between these clades (3.5–6.5%) suggests that the species diversity of *Hypopachus* may be underestimated. These sequence divergence values are lower than the divergences (6.9–7.1%) we noted between sister taxa of *Gastrophryne*, but comparable to or exceed mitochondrial gene divergences noted for valid New World anuran species of *Incilius* (Mendelson et al., 2005), *Dendrobates* (Vences et al., 2003), *Rana* (Pfeiler and Markow, 2008), and salamander species in the genera *Bolitoglossa* (Parra-Olea et al., 2004) and *Pseudoeurycea* (Parra-Olea et al., 2005).

Nelson (1974) examined adult morphology, color pattern, and male advertisement call data and found no differences among the lowland populations of *Hypopachus*. Using the data from Nelson (1973a, 1974), we grouped male advertisement call data according to the geographic clades recovered in our phylogenetic analysis, but this call data alone failed to distinguish the clades (Fig. 4). Examination of color photographs, field note remarks, X-rays, and preserved adult specimens (EG, unpubl. data) showed substantial variation (Fig. 5), and failed to identify any morphological or color pattern characters that could unequivocally distinguish the lowland clades of *Hypopachus*. Preliminary examination of tadpoles from different clades (ENS, unpubl. data) suggest there might be morphological differences that can distinguish the lineages, but available sample sizes are too low to determine whether these differences are intraspecific variation or diagnostic characters among valid evolutionary lineages. Because there is currently no morphological, mensural or meristic character to diagnose the western Mexico (WMX) clade from other lowland populations (MML) of *Hypopachus*, we choose to uphold current taxonomy and consider all lowland populations as one species, *H. variolosus*.

4.2. Outgroup relationships

Our well-supported relationship ((*Hypopachus* + *Gastrophryne*) *Elachistocleis*) is consistent with the studies of Frost et al. (2006), Van Bocxlaer et al. (2006), and Van der Meijden et al. (2007), including the latter authors' reanalyzed molecular data of Frost et al. (2006). Our placement of a well-supported, monophyletic *Chiasmocleis* as sister to the *Hypopachus*/*Gastrophryne*/*Elachistocleis* lineage is also consistent with the nuclear-gene phylogeny of Van der Meijden et al. (2007).

The distinct placement of *Gastrophryne usta* in our analyses was unexpected. Only one previous study has focused on the evolutionary genetics of the genus *Gastrophryne* (Makowsky et al., 2009), but most of their sampling was limited to *G. carolinensis* (the type species of the genus) in the eastern United States, which is remarkable for its lack of genetic diversity, possibly the consequence of a recent bottleneck. The uncorrected *p* mitochondrial sequence divergence between *G. usta* and the *G. carolinensis*/*olivacea* clade (14.3%) is comparable to divergences between both lineages of *Gastrophryne* to either *Hypopachus* (9.5–14.6%) or *Elachistocleis* (12.7–13.8%). *Gastrophryne usta* is known from open tropical deciduous formations, marshes, savannas, and dry forest from central Sinaloa, Mexico to El Salvador on the Pacific versant, and Veracruz to Oaxaca, Mexico on the Atlantic versant (Nelson, 1972a; Köhler et al., 2006; Frost, 2011). Nelson (1972b) and Köhler et al. (2006) provided accounts of this species' ecology, morphometrics, and natural history. Nelson (1972a) and Nelson and Altig (1972) noted that *G. usta* is distinguished from its congeners by the possession of paired metatarsal tubercles (single in congeners) in adults and tadpoles with well-developed hind limbs, presumably an adaptation for xeric habitats. Nelson and Altig (1972:382) noted that *Hypopachus* also have two metatarsal tubercles, but tadpoles in this genus are distinguished from all *Gastrophryne* by having scalloped or papillate labial flap margins and relatively longer, ventrally convergent or overlapping labial flaps. Compared to its congeners, Nelson (1972a, 1973b) also noted a markedly lower harmonic interval (70–130 Hz [range includes data from Fouquette and Rossman, 1963]) in the male advertisement call for *G. usta* (155–280 Hz in other species, call of *G. pictiventris* not quantified [Nelson, 1972c,

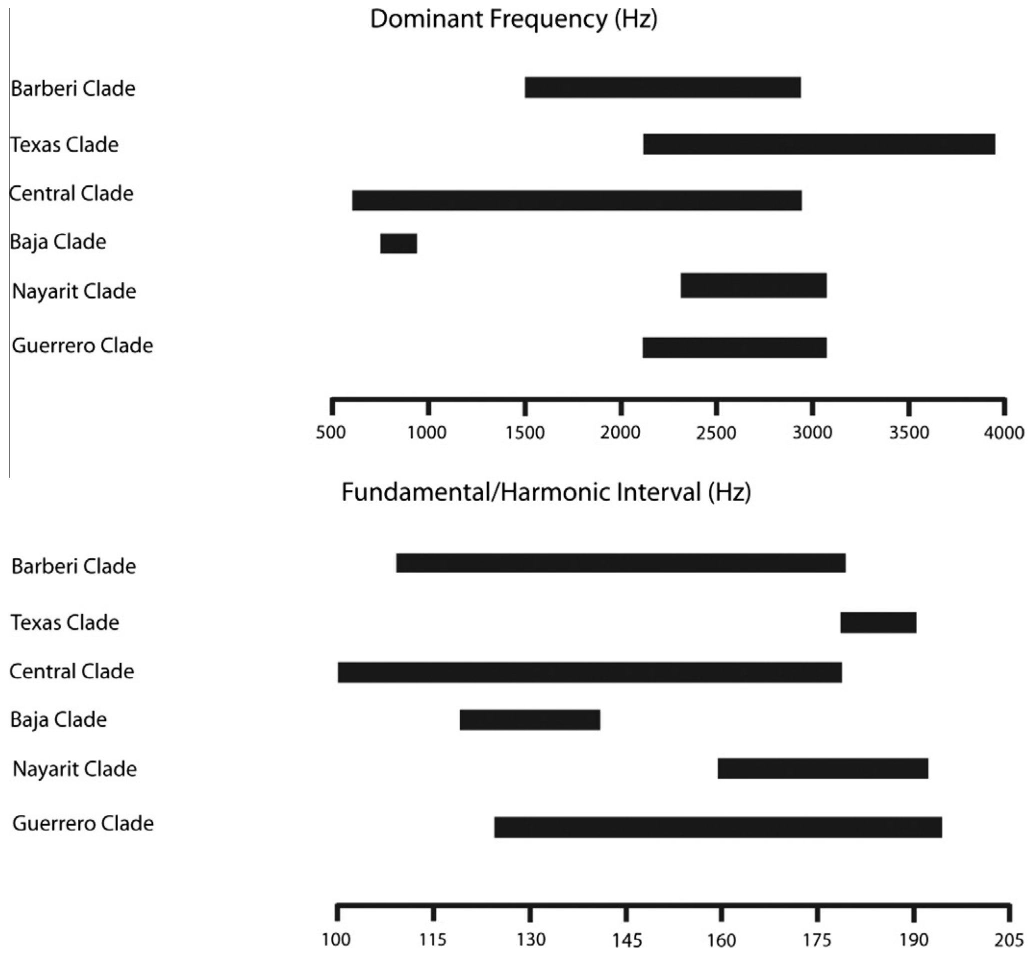

Fig. 4. Graphical representation of frequencies and fundamental/harmonic intervals of well-supported clades of *Hypopachus*. Data are summarized from Nelson (1973a, 1974).

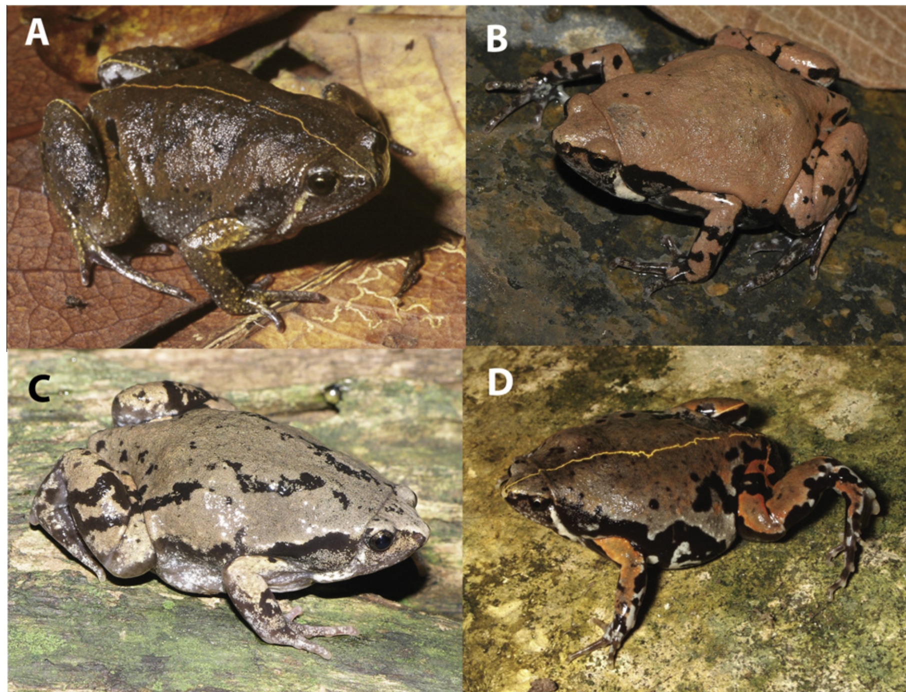

Fig. 5. Photographs of selected specimens of *Hypopachus* in life, showing extensive color pattern variation. (A) *H. barberi* (UTA A-50963) from El Quiché, Guatemala; (B) *H. variolosus* (UTA A-60367) from Guerrero, Mexico; (C) *H. variolosus* (UTA A-60368) from Morelos, Mexico; (D) *H. variolosus* (UTA A-60393) from Petén, Guatemala.

1973b; Savage, 2002]), and a salient difference in calling behavior—*G. usta* calls from concealed locations under leaves, grass, or trash, either at the edge of water or as much as 10 m away, whereas all other *Gastrophryne* species typically call in water with the body floating freely, but submerged from the axillae posteriorly. Because our outgroup sampling was not designed to test Gastrophryninae relationships, it would be premature to suggest a taxonomic rearrangement for *G. usta*, but future studies should examine more samples of this taxon in an expanded phylogenetic study of gastrophrynines.

Our strong support for the inclusion of *Phrynomantis* in the Gastrophyninae and moderate support (81% MP, 89% ML, 1.0 BI) for a sister relationship of *Phrynomantis microps* to *Chiasmocleis* is a novel finding. Other studies either recovered *Phrynomantis* in a well-supported and separate lineage from Gastrophryninae (Frost et al., 2006; Van Bocxlaer et al., 2006), or as an unresolved basal microhylid lineage (Van der Meijden et al., 2007), a paradox that the latter authors explained as "a biological polytomy, due to a fast initial radiation."

4.3. Historical biogeography

A South American origin for the ancestor of *Hypopachus* was suggested by Van der Meijden et al. (2007), who commented that *Hypopachus* and *Gastrophryne* were firmly nested within their South American gastrophrynine clade, and their divergence estimate for the *Hypopachus*/*Gastrophryne* lineage was 17 (±4 standard deviation) mya. Several fossils of *Gastrophryne*, some identified to extant species, are known in North America – *Gastrophryne carolinensis* and *G. olivacea* have been identified from Pleistocene (<2 mya) deposits in Florida (Holman, 1965, 1996) and Texas (Holman, 1963), respectively. The presence of a *Gastrophryne* cf. *carolinensis* ilium fossil from Hemmingfordian Miocene deposits of Florida (Auffenberg, 1956; Holman, 1965) suggests the microhylid invasion of North America occurred at least 18 mya. This predates the mid-Miocene (ca. 15 mya) island-arc system that might have made contact with South America (Duellman, 2001) to allow a land-based invasion of the *Gastrophryne*-like ancestor from South America. It is possible that *Gastrophryne* could have rafted to Central America and further north before the Pliocene (ca. 3.5–5 mya) contact between North and South America via the Panama isthmus – this hypothesis seems likely given the Florida fossil, the basal position of the northern species of *Gastrophryne* relative to *G. usta* and *Hypopachus*, the Van der Meijden divergence estimate of 17 mya, and the increasing number of studies that have demonstrated trans-oceanic dispersal in amphibians (e.g., Heinicke et al., 2007; Measey et al., 2007; Vences, 2004). Because many microhylids (including *Hypopachus* and *Gastrophryne*) are semi-fossorial burrowers with secretive habits and brief reproductive seasons, these frogs may be especially well-suited for trans-oceanic transport inside floating flotsam.

Given the lack of morphological, color pattern, and call data that can be used to diagnose our genetically defined lineages of *Hypopachus*, the relatively modest sequence divergence between lowland sister clades, and the late Miocene/early Pliocene divergence estimates, it is likely that *Hypopachus* lineages formed relatively recently in geological time. However, given the high haplotype diversity, negative Fu's *F*s values, and multimodal distribution shapes in the examined mismatch distributions, it is also likely that the clades have not experienced secondary contact since their initial divergence. Some features of the distribution, habitat preference, and phylogenetic position of *Hypopachus* in this study are consistent with known events of Middle American geological history. Campbell (1999) noted that it is likely that many montane species in Middle America (e.g., *H. barberi*) did not exist until the late Miocene (ca. 7 mya) at the earliest because mountains were just forming at this time; the high elevations currently associated with Nuclear Central America did not occur until the Pliocene 2–3 million years later. Campbell (1999) and Duellman (2001) added that vertical oscillations in climate during the Pleistocene ($\leqslant$1.8 mya) resulted in depression of vegetational belts (perhaps as much as 1000 m). These depressions likely led to connections between montane cloud forests, which would explain the close relationship among currently disjunct highland populations of *H. barberi.*

Another consistent link between *Hypopachus* phylogeny and geological history involves the slightly divergent, well-supported lowland lineage we recovered from our Baja Verapaz and Zacapa samples (Baja Clade), which are restricted to xeric interior Guatemalan valleys. Campbell (1999) discussed the formation of the Salamá Valley during the mid- to late Pliocene (ca. 2.5–3.6 mya) when the headwaters of the Río Polochic were captured by the Río Negro. Later in the Pleistocene (<2 mya), renewed volcanic activity in southwestern Guatemala influenced local weather patterns to produce rain shadow conditions in this interior valley, again suggesting relatively recent events in the evolution of *H. variolosus*.

Explaining the disjunct distribution of the divergent lowland clades of *Hypopachus* in Mexico is more problematic, but there seem to be similarities with distribution and phylogeographic patterns in other amphibian and reptile groups. There is an interesting parallel to the pattern we found in *Hypopachus* and the distributions of sister taxa in the monotypic hylid genera *Diaglena* and *Triprion* – *Diaglena* occurs in the Pacific lowlands of Mexico from Sinaloa to Oaxaca, whereas *Triprion* inhabits the xeric habitats of the Yucatán Peninsula (Duellman, 2001; Frost, 2011). It is likely that a common ancestor to each hylid genus was confined to the subhumid habitats of Middle America and once inhabited a more widespread area, but subsequently was confined to relict populations during changes in aridity from glacial oscillations of the Pleistocene. In a study of the Neotropical snake genus *Leptodeira*, Daza et al. (2009) recovered similar patterns of genetic divergence in Pacific versant Mexico populations of *L. maculata* and *L. annulata cussiliris*. These authors explained this pattern as a possible consequence of Pleistocene sea level fluctuations in the Isthmus of Tehuantepec, which may have isolated vertebrate populations in the lowlands of Mexico. A second, older explanation from the snake study is either the formation of main river basins or Miocene climatic changes, which is consistent with our divergence estimates for *Hypopachus*.

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