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## ***Gambusia quadruncus* (Cyprinodontiformes: Poeciliidae): a new species of mosquitofish from east-central México**

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*Gambusia quadruncus* n. sp., the llanos mosquitofish, is described from east-central México. The region inhabited by the species represents a hotspot of diversity of *Gambusia*, and *G. quadruncus* sometimes coexists with at least three congeners. The species differs from its closest relative, *Gambusia affinis*, in several characteristics with plausible effects on reproductive isolation, e.g. body size, body and fin morphology, male genital morphology (distal tip of gonopodium) and female anal spot morphology (colouration near the urogenital sinus). Moreover, combined analysis of mitochondrial and nuclear gene sequence data (c. 2158 total base pairs) indicates reciprocal monophyly of *G. quadruncus* and its sister species *G. affinis*, with levels of genetic divergence suggesting the two species diverged from one another over a million years ago. The origin of *G. quadruncus* may reflect a vicariant event associated with Pliocene orogenesis in the Tamaulipas Arch and a frontal section of the Sierra Madre Oriental (Lleran Mesas). *Gambusia quadruncus* inhabits a variety of freshwater habitats across several river drainages, with its range spanning at least 350 km from north to south, covering over 25 000 km<sup>2</sup>. A key to aid identification of the species is provided.

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### INTRODUCTION

The new world livebearers (Family Poeciliidae, Rosen & Bailey, 1963; or subfamily Poeciliinae, Parenti, 1981), comprise a diverse group of fishes (>220 species; Lucinda, 2003) originating c. 68 million years ago (Hrbek *et al.*, 2007). During this time, these fishes successfully colonized a remarkably diverse range of environments (e.g. oceans, estuaries, lakes, rivers and springs), and occupied many isolated regions,

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such as islands, river drainages separated by mountains, and extreme habitats surrounded by expanses of unsuitable environments (*e.g.* caves and sulphur springs). Today, poeciliids inhabit most types of aquatic habitats available in South, Central and North America, and the Caribbean (excluding the closely related subfamilies Aplocheilichthyinae and Procatopodinae, which include African representatives).

*Gambusia* is the most speciose genus in the family Poeciliidae, including 42 valid described species and a number of undescribed species. They are small fishes (typically <60 mm standard length,  $L_S$ ), distributed from northern Colombia to the central and south-eastern U.S.A., and across numerous Caribbean Islands (Rauchenberger, 1989). As might be expected, speciation in the genus appears to involve both ecology and geographical isolation (Rauchenberger, 1988; Langerhans *et al.*, 2007). In this study, a new species of *Gambusia* is described from east-central México (Fig. 1). Twenty species of *Gambusia* are known to occur in México, with seven inhabiting this region [*Gambusia affinis* (Baird & Girard 1853), *Gambusia atrora* Rosen & Bailey 1963, *Gambusia aurata* Miller & Minckley 1970, *Gambusia panuco* Hubbs

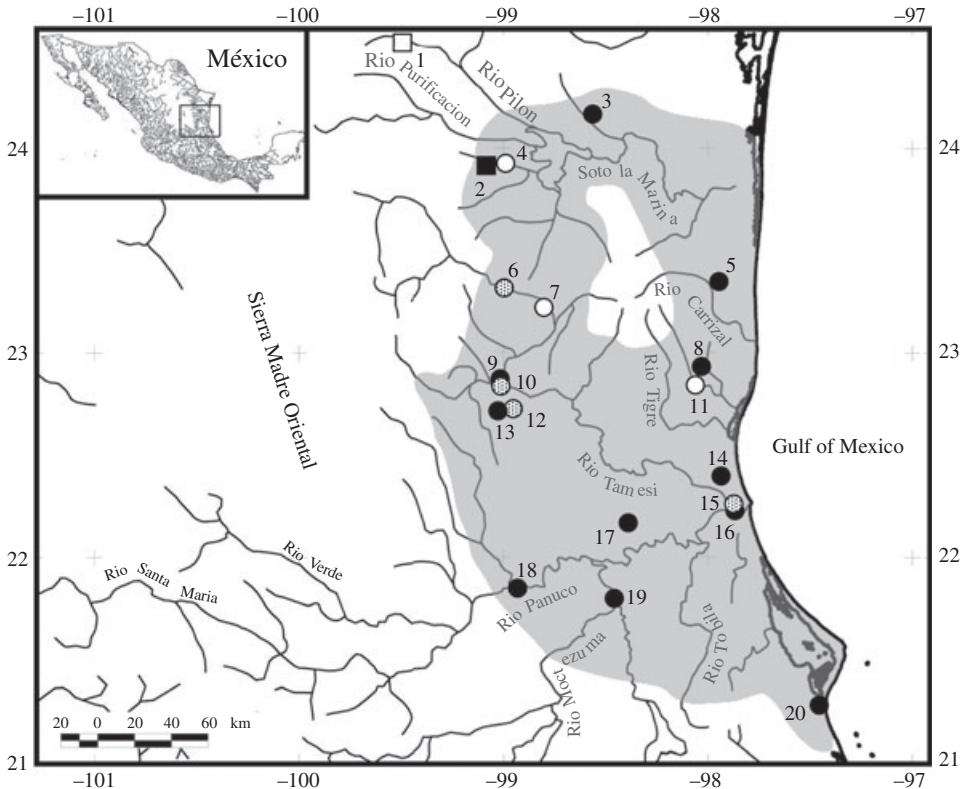


FIG. 1. Locations of collections of *Gambusia quadruncus* examined in the study, with an estimated range of the species in east-central México (□). The gap within the range reflects the apparent absence of *G. quadruncus* from the higher elevations of the Sierra de Tamaulipas. The two collections of *Gambusia affinis* from México included in analyses in this study are also noted. ●, ○, ○, *G. quadruncus*; ■, □, *G. affinis*; ●, ■, morphological datasets; ○, □, molecular datasets; ○, □, both morphological and molecular datasets. Collection numbers refer to those listed in Appendices I and II.

1926, *Gambusia regani* Hubbs 1926, *Gambusia speciosa* Girard 1859 and *Gambusia vittata* Hubbs 1926]. Considering the number of co-occurring species of *Gambusia*, and the ongoing work suggesting a number of additional, undescribed congeners in this region (R. B. Langerhans, M. E. Gifford, C. Pedraza-Lara, O. Domínguez-Domínguez, I. Doadrio, unpubl. data), east-central México appears to represent a major hotspot of diversity of *Gambusia*. The new species inhabits a range of freshwater habitats (e.g. lakes, large rivers and swiftly flowing spring-fed streams), often coexisting with other congeners.

## MATERIALS AND METHODS

For the description of *Gambusia quadruncus*, numerous types of data were examined: body size, fin-ray and scale meristics, body and fin morphology, morphology of the distal tip of the gonopodium (modified anal fin in males, functioning as a copulatory organ), body colour, number of vertebrae, number of modified haemal spines in the anourogenital region of males (sometimes called gonapophyses; Rosen & Gordon, 1953; Rosen & Bailey, 1963) and DNA sequence data. Fin-ray, scale and gonopodial counts follow Greenfield (1983); counts of vertebrae and modified haemal spines were made from X-ray radiographs following Greenfield (1983); gonopodial terminology follows Rosen & Bailey (1963); measurement of relative length of serrae follows Peden (1973a) and description of colour patterns follows traditional methodology and terminology (Minckley, 1963; Peden, 1973a; Greenfield, 1983). Institutional abbreviations follow Fricke & Eschmeyer (2012). For gonopodial morphology, numerical codes are used for the following characters. Ray 4a elbow location: 0 = elbow distal to ray 4p serrae by less than one segment, 1 = elbow distal to ray 4p serrae by more than or equal to one segment but less than two and 2 = elbow distal to ray 4p serrae by two or more segments. Gap height between rays 4a and 4p distal to elbow: 0 = absent, 1 = small (height less than height of first segment distal to elbow), 2 = medium (height approximately equal to height of first segment distal to elbow) and 3 = large (height greater than height of first segment distal to elbow). Gonopodial terminus shape: 0 = acute tip, 1 = slightly upturned tip and 2 = terminal hook present.

Meristic and morphological measurements were conducted on 27 adult male and 31 adult female specimens (*i.e.* holotype, allotype and paratypes) using digital photographs, stereo microscopy and radiographs. To visualize the skeletal morphology of the gonopodium, a micro X-ray computed-tomography scan of the gonopodium of a single male specimen was conducted at the HRXCT Facility at the University of Texas at Austin (Ketcham & Carlson, 2001). This procedure yielded a three-dimensional reconstruction of the bony elements of the gonopodium. For body size, body and fin morphology, gonopodial-tip morphology and body colour, a larger sample size was employed, and comparisons of *G. quadruncus* and its putative sister species, *G. affinis* are provided (115 male and 155 female *G. quadruncus*, 116 male and 118 female *G. affinis*). Additional material was examined for all putatively sympatric congeners (*G. speciosa*, *G. panuco*, *G. regani*, *G. aurata* and *G. vittata*), and other putatively close relatives [additional species in the *affinis* species group of Rauchenberger (1989): *Gambusia holbrooki* Girard 1859, *Gambusia lemaitrei* Fowler 1950] but all are highly divergent and easily distinguished from *G. quadruncus* and thus direct comparisons to these species are not presented here. An identification key to distinguish the new species from similar congeners, however, is provided. Except for body colour and vertebral count, adult males were exclusively examined for comparisons among species, as body size remains relatively constant subsequent to sexual maturity in males but not in females (Turner, 1941; Johnson, 1976; Hughes, 1986; Yan, 1987), several traits of interest are found only in males (e.g. gonopodium and modified haemal spines) and identification and systematic research in poeciliids typically focuses on males, and this avoids possible effects of pregnancy on body shape. Specimens from 15 collections of *G. quadruncus* (one collection contained no males) and 13 collections of *G. affinis* were examined (Appendix I). Body size was measured as  $L_S$ . For body and fin morphology, seven morphometric traits were measured (see last

seven rows in Table I).  $\log_{10}$ -transformed residual values were calculated for each trait from regressions on  $\log_{10}$ -transformed  $L_S$  prior to analysis. To provide intuitive metrics for species differences in morphology, per cent differences in shape variables between species were calculated by back-transforming the species means to mm and dividing the larger value by the smaller value (*i.e.* per cent differences in mm lengths). For gonopodial-tip morphology, nine characters were examined (Table II). Preserved body colour was assessed for adult male and female specimens in the 28 collections examined here, and live body colour was based on field notes and photographs of live animals from six populations of *G. quadruncus* and 11 populations of *G. affinis*.

Differences between *G. quadruncus* and *G. affinis* in body size, body and fin morphology and gonopodial-tip morphology were tested using nested ANOVA. In each case, the model tested for effects of species and population nested within species (random factor). This analytical design (mixed-model nested ANOVA) effectively treats population as the unit of replication. For body size,  $\log_{10}$ -transformed  $L_S$  was used as the dependent variable. For body and fin morphology (seven  $\log_{10}$ -transformed residual characters) and gonopodial-tip morphology (nine characters), separate principal component analyses (PCA) were first conducted using correlation matrices to reduce dimensionality, and then principal component (PC) axes that explained more variation than expected under a broken-stick model were retained (Jackson, 1993). These PC scores were then used as dependent variables in nested ANOVAs to test for differences in body and fin morphology and gonopodial-tip morphology. To provide an intuitive metric of the overall distinctiveness of the two species (*i.e.* percentage of fishes correctly classified to species), a discriminant function analysis (DFA) was conducted for two datasets (1) body size and body and fin morphology ( $\log_{10}$ -transformed  $L_S$  and the seven residual shape variables) and (2) gonopodial-tip morphology (all nine gonopodial characters). DFAs were conducted using jackknife sampling as a cross-validation technique (*i.e.* each individual was sequentially removed from the dataset and classified according to a discriminant function derived with the remaining data).

To assess genetic divergence and phylogenetic relationships among *G. quadruncus* and its close relatives, mtDNA and nDNA gene sequences were obtained for *G. quadruncus*, its presumably most closely related congeners based on morphology (*G. affinis* and *G. holbrooki*), other potentially close relatives based on prior species-group assignment (*G. aurata*, *G. speciosa* and *G. lemaitrei*), and two outgroup taxa [*Heterophallus rachovii* (= *Gambusia rachovii*) Regan 1914 and *Belonesox belizanus* Kner 1860] (see Appendix II for locality information and sample sizes for each species and each gene). One specimen per population was examined: 22 populations for two mtDNA genes; subset of 16 populations for one nDNA gene. For *G. quadruncus* and its closest relatives, DNA sequences from multiple populations across their range were examined, while a single specimen for other species was examined. For mtDNA, PCR was used to amplify a 975 bp fragment of the NADH subunit 2 gene (ND2) and a 402 bp fragment of the cytochrome *b* gene (*cyt b*). For ND2, the primers L3975 (5'-AAG CTT TCG GGC CCA TAC CC-3') and H5099 (5'-GCT TAG GGC TTT GAA GGC CC-3') were used, where the letters in the primer names represent the light (L) and heavy (H) strand, and the numbers indicate their 5' position in the mitochondrial genome of *G. affinis* (Miya *et al.*, 2003). PCR conditions included an initial denaturation at 95° C for 180 s followed by 30 cycles of denaturation at 95° C for 30 s, annealing at 56° C for 30 s and extension at 70° C for 90 s; concluding with a final extension at 70° C for 240 s. For *cyt b*, primers and conditions described in Lydeard *et al.* (1995) were used. For nDNA, a fragment of the first intron of the S7 ribosomal protein gene (S7) was amplified following methods described in Chow & Hazama (1998), with the modification of the PCR conditions to accommodate a 67-57 touchdown: the annealing temperature in the first step was 67° C, dropped 1° C with each subsequent cycle until it reached 57° C, which was maintained for an additional 24 cycles. The size of the S7 intron fragment ranged from 743 to 774 bp, with the aligned length being 781 bp including gaps. Sequences were aligned by eye. All sequence data have been deposited in GenBank.

For molecular analyses, evidence for reciprocal monophyly among *G. quadruncus* and its closest relatives was the primary concern. Confirmatory evidence would suggest that each species has maintained separate gene pools for a substantial period of time. Phylogenetic relationships were inferred from DNA sequences using maximum-likelihood (ML) and Bayesian

TABLE I. Morphometric measurements and counts for *Gambusia quadricus* type specimens (27 males and 31 females). Measurements are in mm. The mode is presented for integer measurements, while the mean is provided for all measurements

Character	Holotype	Males			Females		
		Mode	Mean $\pm$ s.d.	Range	Mode	Mean $\pm$ s.d.	Range
$L_S$	16.07	–	17.25 $\pm$ 1.94	14.2–21.6	–	21.12 $\pm$ 3.12	16.0–27.9
Dorsal rays	7	7	7.04 $\pm$ 0.19	7–8	7	7.16 $\pm$ 0.45	7–9
Anal rays	–	–	–	–	10	9.97 $\pm$ 0.31	9–11
Pelvic rays	6	6	6.00 $\pm$ 0.00	6–6	6	6.00 $\pm$ 0.00	6–6
Pectoral rays	12	12	12.26 $\pm$ 0.45	12–13	13	12.68 $\pm$ 0.54	12–14
Branched caudal rays	12	12	12.04 $\pm$ 0.61	10–13	12	12.47 $\pm$ 0.90	10–14
Lateral scales	31	31	30.70 $\pm$ 0.61	29–31	31	30.52 $\pm$ 0.68	29–32
Predorsal scales	17	16	16.41 $\pm$ 0.50	16–17	15	15.61 $\pm$ 0.76	14–17
Vertebrae	32	32	32.04 $\pm$ 0.52	31–33	32	31.58 $\pm$ 0.76	30–33
Modified haemal spines	3	3	3 $\pm$ 0.00	3–3	–	–	–
Head depth $L_S^{-1}$	0.18	–	0.18 $\pm$ 0.01	0.17–0.20	–	0.21 $\pm$ 0.01	0.19–0.23
Head length $L_S^{-1}$	0.28	–	0.28 $\pm$ 0.01	0.25–0.29	–	0.29 $\pm$ 0.02	0.26–0.33
Caudal-peduncle depth $L_S^{-1}$	0.15	–	0.16 $\pm$ 0.01	0.14–0.18	–	0.15 $\pm$ 0.01	0.14–0.16
Predorsal length $L_S^{-1}$	0.63	–	0.61 $\pm$ 0.02	0.58–0.64	–	0.68 $\pm$ 0.01	0.65–0.72
Postanal length $L_S^{-1}$	0.45	–	0.44 $\pm$ 0.02	0.41–0.48	–	0.32 $\pm$ 0.02	0.28–0.36
Caudal fin length $L_S^{-1}$	0.25	–	0.24 $\pm$ 0.01	0.21–0.27	–	0.24 $\pm$ 0.01	0.22–0.28
Gonopodium length $L_S^{-1}$	0.33	–	0.34 $\pm$ 0.02	0.32–0.39	–	–	–

$L_S$ , standard length.

TABLE II. Gonopodial characters for male *Gambusia quadruncus* and *Gambusia affinis*. The mode is presented for integer measurements, while the mean is provided for all measurements

Character	<i>G. quadruncus</i>			<i>G. affinis</i>			
	Holotype	Mode	Mean $\pm$ s.d.	Range	Mode	Mean $\pm$ s.d.	Range
Ray 4p serrae number	4	4	3.67 $\pm$ 0.54	3–5	4	4.57 $\pm$ 0.76	3–6
Ray 4p serrae relative length	1.5	–	1.49 $\pm$ 0.27	0.9–2.0	–	1.85 $\pm$ 0.31	1.2–2.3
Segments distal to ray 4p serrae	10	10	9.91 $\pm$ 0.81	8–11	9	8.95 $\pm$ 0.75	7–10
Ray 4a elbow location	1	1	1.05 $\pm$ 0.49	0–2	0	0.00 $\pm$ 0.00	0–0
Fused elbow elements	2	2	2.28 $\pm$ 0.45	2–3	2	2.48 $\pm$ 0.51	2–3
Segments distal to ray 4a elbow	6	7	6.70 $\pm$ 0.89	5–9	8	7.34 $\pm$ 0.96	6–9
Gap between rays 4a and 4p distal to elbow	3	3	2.83 $\pm$ 0.43	1–3	1	1.35 $\pm$ 1.10	0–3
Ray 3 spine number	10	10	9.86 $\pm$ 1.05	7–12	11	11.14 $\pm$ 1.17	9–14
Terminus shape	2	2	1.95 $\pm$ 0.21	1–2	0	0.39 $\pm$ 0.49	0–1

inference (BI) approaches. mtDNA sequences were concatenated for analysis (after first confirming homogeneity of phylogenetic signal of the two genes; partition homogeneity test,  $P > 0.05$ ), and S7 sequences were analysed both separately and combined with mtDNA sequences (partition homogeneity test for mtDNA and nDNA datasets,  $P > 0.05$ ). A total of seven data partitions were employed: one for each codon position of the ND2 and *cyt b* genes, and one for S7. ML phylogenetic relationships were estimated using PAUP 4.0b10 (Swofford, 2003), with the optimal maximum-likelihood model of DNA sequence evolution determined for each of the three datasets (mtDNA, S7 and combined) using the Akaike information criterion (AIC) with jModelTest 0.1.1 (Posada, 2008). Rates were optimized separately for each data partition. The ML heuristic search employed 10 replicate random-sequence stepwise additions for starting trees and tree bisection and reconnection branch swapping. To estimate support for nodes in the ML trees, 100 bootstraps of sequence data were generated, preserving partitioning structure using RAxML 7.03 (Stamatakis, 2006). BI relationships were estimated using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003), with the optimal maximum-likelihood model of sequence evolution determined as above for each of the seven data partitions. Partitioned mixed-model Bayesian analyses were performed, where each data partition was assigned its own evolutionary model, with model parameter values being unlinked among partitions assigned the same molecular evolutionary model. MrBayes 3.1.2 was run for 5 000 000 generations, sampling trees every 100 generations. The lower 25% of the trees were discarded as burn-in trees in the computation of a 50% majority rule consensus tree. Support values for inferred clades were calculated from Bayesian posterior probabilities.

Specimens from numerous collections were examined from the University of Michigan Museum of Zoology (UMMZ), the Texas Natural History Collection (TNHC) and the Colección de Peces de la Universidad Michoacana de San Nicolás de Hidalgo (CPUM) in Morelia, México, to better approximate the species' distribution and habitat use.

## RESULTS

### *GAMBUSIA QUADRUNCUS* SP. NOV. LANGERHANS

Llanos mosquitofish (Figs 2 and 3 and Tables I and II).

#### *Holotype*

UMMZ 248855, male, 16.1 mm  $L_S$ , collected on 22 June 2005 by R. B. Langerhans, T. J. DeWitt and D. García-Bedoya from Río Guayalejo at El Limón, Tamaulipas, México, 19 km south of Xicoténcatl (22° 49' 53" N; 99° 0' 39" W) (Figs 2 and 4).

#### *Allotype (and paratype)*

UMMZ 248856, female, 25.0 mm  $L_S$ , taken with the holotype.

#### *Paratypes*

All from Tamaulipas, México. TNHC 45833, one specimen, 25.4 mm  $L_S$ , taken in Laguna de Chairrel at western edge of Tampico (22° 15' 2" N; 97° 53' 17" W), same collectors and date as holotype; TNHC 45834, 22 specimens, 16.3–23.8 mm  $L_S$ , taken at the Highway 85 crossing of a north-flowing canal at the eastern edge of Ciudad Mante (22° 43' 31" N; 98° 57' 21" W), same collectors and date as holotype; TNHC 45835, five specimens, 15.2–24.1 mm  $L_S$ , taken in Río Guayalejo at the Highway 85 crossing near Llera (23° 18' 52" N; 99° 0' 11" W), collected

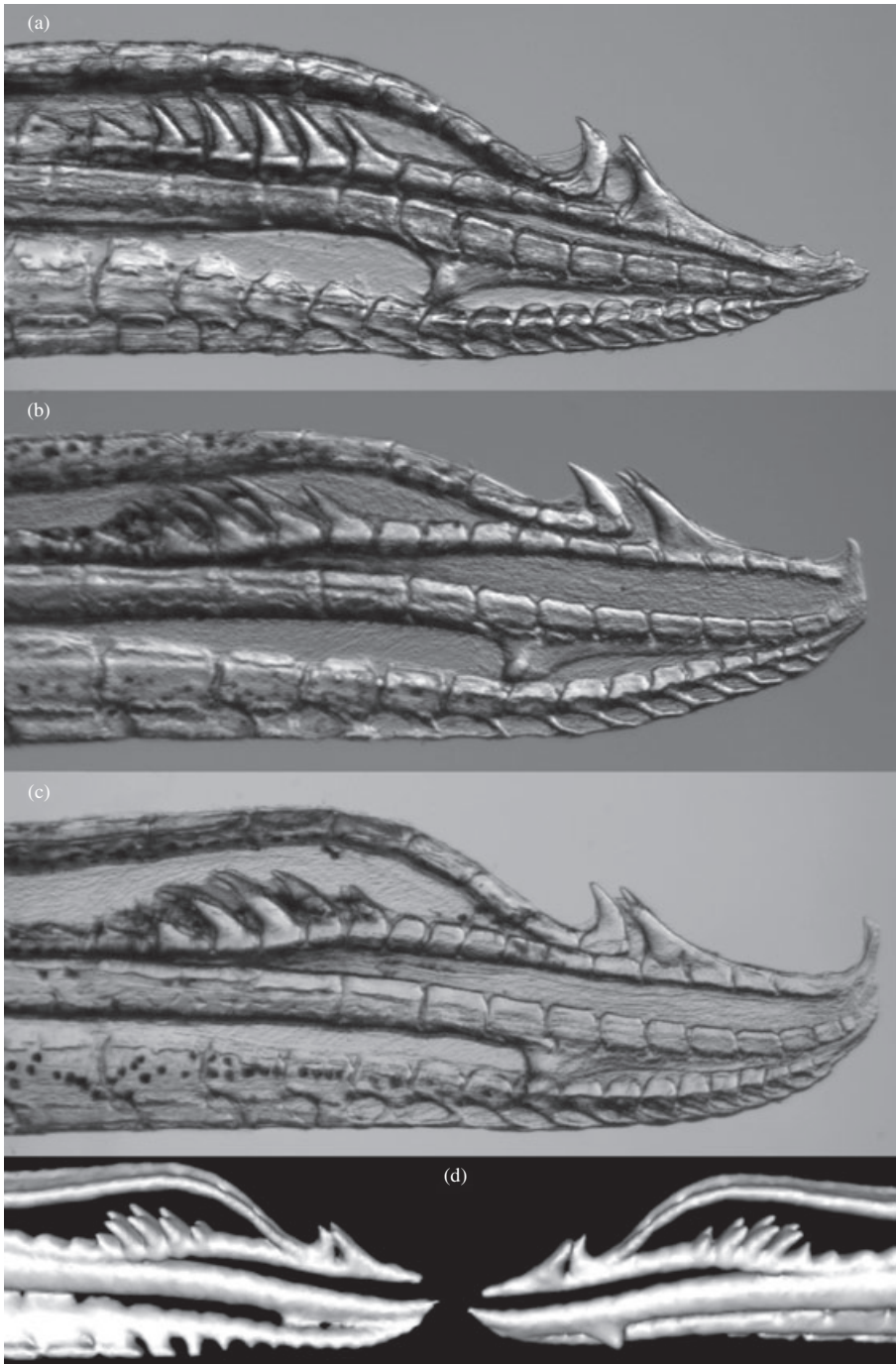


FIG. 2. Lateral view of the distal tip of the gonopodium for (a) *Gambusia affinis*, LLSTC 08665, 19.9 mm standard length ( $L_S$ ), (b) *Gambusia quadruncus*, holotype, UMMZ 248855, 16.1 mm  $L_S$ , (c) *G. quadruncus*, paratype, TNHC 45834, 20.9  $L_S$  and (d) same specimen as in (c) but visualized with X-ray computed tomography for the left and right sides of the gonopodium (dorsolateral perspective).





FIG. 3. *Gambusia quadruncus*, paratypes, (a) UMMZ 248858, male, 19.9 mm standard length ( $L_S$ ) and (b) UMMZ 248858, female, 27.9 mm  $L_S$ .

on 23 June 2005 by the same collectors as holotype; UMMZ 248857, 26 specimens, 14.2–27.0 mm  $L_S$ , taken with the holotype; UMMZ 248858, two specimens, 19.9–27.9 mm  $L_S$ , taken in an irrigation ditch on the west side of Highway 85 c. 1.6 km north of El Limón (c. 22° 50' 7" N; 99° 1' 24" W), collected on 28 February 1963 by R. R. Miller and R. J. Schultz.

#### Diagnosis

A member of the *affinis* species group as defined by Rauchenberger (1989) is based on the small, stout spines at the distal end of the third gonopodial ray, the lateral bulge at the anteroproximal tip of the elbow on gonopodial ray 4a and the scalloped distal tip of the fifth pectoral fin ray in males. *Gambusia quadruncus* is distinguished from all other members of the *affinis* species group by the following combination of characters: terminal hook at the distal tip of the gonopodium formed by rays 4a, 4p or both; elbow on gonopodial ray 4a positioned more distal relative to serrae (typically positioned one segment or more distal to serrae) and anal spots in females that are permanent, more prominent and positioned more posteriorly than other members (typically spanning the second to fifth or sixth rays).

#### Description

Overall, *G. quadruncus* is one of the smaller species of *Gambusia* with a moderately deep body, large head, light-orange unpaired fins and an abundance of semi-prominent spots on the caudal fin; males exhibit a short, highly distinctive gonopodium and females exhibit prominent anal spots on the body posterodorsal to the insertion of the first anal fin ray. While male body size is variable, *G. quadruncus*



FIG. 4. Río Guayalejo in El Limón, Tamaulipas, México, type locality of *Gambusia quadruncus*. The species was collected at the margins of water lilies (*Nymphaea ampla*) and other vegetation (e.g. *Ceratophyllum*, *Hydrilla* and *Eleocharis*) in the calm, backwater section of the river depicted here.

appears to represent one of the smallest species of *Gambusia*, with adult males examined here averaging *c.* 18 mm  $L_S$ . The gonopodium of *G. quadruncus* possesses a hooked terminus at the distal end. That is, like most species of *Gambusia*, *G. quadruncus* has a terminal hook on ray 5a, followed by a compound hook on ray 4p situated just distal to the 5a hook. Unlike any other species of *Gambusia*, *G. quadruncus* additionally has a terminal hook at the distal tip of the gonopodium, formed by ray 4p, ray 4a or both. High-resolution CT scanning reveals the terminal hook is formed by soft tissue and not bony elements [see Fig. 2(d), which exclusively depicts bony elements and lacks the terminal hook].

Morphometric and meristic data for types are given in Table I. Gonopodial characters are given in Table II and illustrated in Fig. 2. The general appearance is illustrated in Fig. 3 and the colour patterns are presented in Table III.

Fin rays are as follows: seven dorsal rays (rarely eight or nine), 10 anal rays (females; rarely nine or 11), six pelvic rays, 12 or 13 pectoral rays (rarely 14) and 12 or 13 branched caudal rays (rarely 10, 11 or 14). Scale counts are 30 or 31 lateral scales (rarely 29, 32) and 15–17 predorsal scales (rarely 14).

Three modified haemal spines in the anourogenital region (on vertebrae 14–16) of males, with the two most posterior spines possessing uncinat processes. Vertebrae 32 in males (rarely 31 or 33) and 31 or 32 in females (rarely 30 or 33).

TABLE III. Comparison of colour patterns of *Gambusia quadranicus* and *Gambusia affinis*

Character	<i>G. quadranicus</i>	<i>G. affinis</i>
Body		
Ground colour	Brown-grey with yellow-tan overtones, iridescent blue-purple reflections, operculum with blue-green iridescence	Brown-grey with yellow-tan overtones, iridescent blue-purple reflections, operculum with blue-green iridescence
Scale pocket margins	Moderate to strongly cross-hatched appearance	Moderate to strongly cross-hatched appearance
Spotting	Several dark spots at junctions of cross-hatchings typically present along midline and above midline	Several dark spots at junctions of cross-hatchings typically present along midline and above midline
Lateral band	Absent to dusky and broad	Absent to dusky and broad
Predorsal streak	Present, variable in intensity and width	Present, variable in intensity and width
Postanal streak	Distinct, thin to broad	Weak to distinct, thin
Subocular bar	Present	Present
Dorsal fin spotting	Scattered or in one to two rows	Scattered or in one to two rows
Dorsal fin margin	Dusky to blackened	Dusky to blackened
Dorsal fin colour	Light orange	Transparent to yellow-orange
Caudal fin spotting	Strong and numerous spots (typically 11–24), sometimes a vertical row is present	Peppered with small melanophores to moderate spotting (stronger in males; typically four to 14), sometimes arranged in a vertical row
Caudal fin margin	Dusky to blackened	No colour to dusky
Caudal fin colour	Light orange, darker orange in males	Transparent to yellow-orange
Anal fin spotting (female)	No spots to several spots	No spots to peppered with small melanophores
Anal fin margin (female)	Dusky to blackened	No colour
Anal fin colour	Light orange in females, orange at base of gonopodium in males	Transparent to yellow-orange in females; transparent to yellow-orange at base of gonopodium in males
Female anal region	Prominent and permanent	No spot to moderately dark spot, varying with ovarian cycle
Anal spot strength	Just dorsal of anal fin, typically spanning second to fifth or sixth rays	Just anterior and dorsal of anal fin, as posterior as third anal fin ray
Anal spot location	Length c. 4% of $L_S$	Length c. 2.5% of $L_S$ when present
Anal spot size	Silvery to white with prominent yellow spot above urogenital sinus and anus	Silvery to white with weak to prominent yellow spot above urogenital sinus and anus
Abdomen colour		

$L_S$ , standard length.

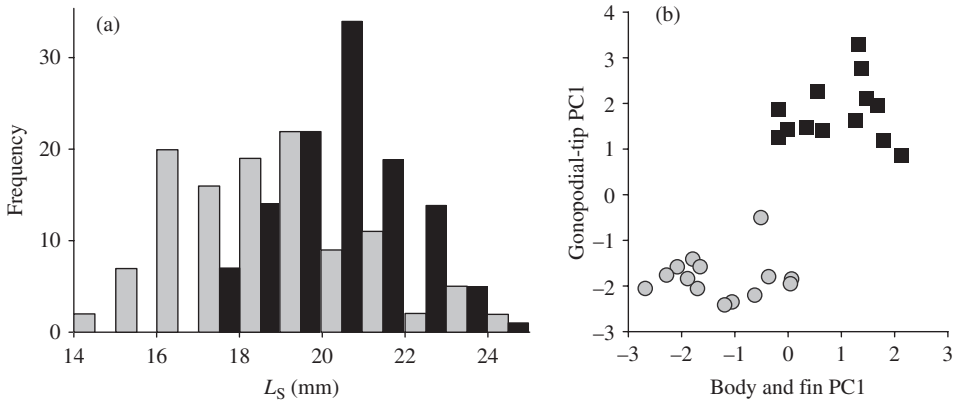


FIG. 5. Differences between *Gambusia quadruncus* and *Gambusia affinis* in male (a) standard length ( $L_S$ ) and (b) body, fin, and gonopodial-tip morphology. Symbols in (b) represent population mean scores along the respective principal component (PC) axis for each collection. (□, ○, *G. quadruncus*; ■, ●, *G. affinis*).

### Comparisons

Among the congeners inhabiting the region of México where *G. quadruncus* is found, only *G. affinis*, *G. speciosa* and *G. aurata* bear notable similarities to *G. quadruncus*, all members of the *affinis* species group as defined by Rauchenberger (1989). Other potentially sympatric congeners (*G. panuco*, *G. regani* and *G. vittata*) are highly distinctive in many respects relative to *G. quadruncus* (notably gonopodial characters and body and fin colouration), and all are distantly related to species in the *affinis* species group based on either prior species-group assignments (Rauchenberger, 1989) or molecular phylogenetic analyses (Lydeard *et al.*, 1995; R. B. Langerhans, M. E. Gifford, C. Pedraza-Lara, O. Domínguez-Domínguez, I. Doadrio, unpubl. data). In M. Rauchenberger's key to Mexican Poeciliidae in Miller *et al.* (2005), *G. quadruncus* keys to number 64, but is then rejected at both 64a (keying to *G. aurata*) and 64b (eventually keying to *G. holbrooki*, *G. speciosa* and *G. affinis*). Thus, an identification key for distinguishing among these five congeners is provided here.

*Gambusia quadruncus* most closely resembles *G. affinis*, but may be distinguished by several characters. First, male *G. quadruncus* tend to be smaller in  $L_S$  than male *G. affinis* [ $F_{1,37.05} = 13.17$ ,  $P < 0.001$ ; Fig. 5(a)]. On average, *G. affinis* is c. 12% larger in  $L_S$  than *G. quadruncus*; however, body size is quite variable in *G. quadruncus*, and three of 14 collections of *G. quadruncus* examined here exhibited mean body sizes that fell within the distribution of mean body size for *G. affinis*. Thus, while differences exist between species in mean standard length, and male body size is known to have a strong genetic basis in a number of poeciliids (Campton & Gall, 1988; Trexler *et al.*, 1990; Campton, 1992; Reznick & Bryga, 1996; Lampert *et al.*, 2010), male body size does not provide a particularly strong diagnostic trait in this case. Second, the two species differ in body and fin morphology [Fig. 5(b)]. Three PC axes were retained for body and fin analysis (Table IV), and species significantly differed along the first two axes (PC1:  $F_{1,32.58} = 29.79$ ,  $P < 0.001$ ; PC2:  $F_{1,36.18} = 6.11$ ,  $P < 0.05$ ; PC3:  $F_{1,37.97} = 0.24$ ,  $P > 0.05$ ). Controlling for  $L_S$ , *G. quadruncus* males have a relatively deeper and longer head (by 7 and 3%,

TABLE IV. Principal component loadings for body and fin morphology and gonopodial tip morphology. Percentages provide the amount of variation explained by each axis. Loadings >0.5 are in bold

Character	PC1	PC2	PC3
Body and fin morphology	31.99%	17.96%	15.87%
Residual log <sub>10</sub> head depth	<b>-0.86</b>	-0.05	0.14
Residual log <sub>10</sub> head length	<b>-0.72</b>	0.34	-0.10
Residual log <sub>10</sub> caudal-peduncle depth	<b>-0.51</b>	<b>-0.60</b>	0.20
Residual log <sub>10</sub> predorsal length	<b>-0.71</b>	0.30	0.23
Residual log <sub>10</sub> postanal length	0.30	-0.35	0.16
Residual log <sub>10</sub> caudal fin length	0.18	-0.13	<b>0.92</b>
Residual log <sub>10</sub> gonopodium length	0.31	<b>0.74</b>	0.35
Gonopodial tip morphology	42.49%	15.28%	12.25%
Ray 4p serrae number	<b>0.73</b>	0.01	0.08
Ray 4p serrae relative length	<b>0.67</b>	0.15	0.30
Segments distal to ray 4p serrae	-0.46	<b>0.70</b>	-0.10
Fused elbow elements	0.27	0.43	<b>0.70</b>
Ray 4a elbow location	<b>-0.83</b>	0.15	0.22
Segments distal to ray 4a elbow	0.42	0.48	<b>-0.65</b>
Gap between rays 4a and 4p	<b>-0.71</b>	0.29	-0.09
Ray 3 spine number	<b>0.64</b>	<b>0.55</b>	0.01
Terminus shape	<b>-0.88</b>	0.14	0.13

respectively), longer predorsal length (by 3%), deeper caudal peduncle (by 7%) and shorter gonopodium (by 5%) than *G. affinis* males. Based on  $L_S$  and body and fin morphology, DFA correctly assigned 97% (138 of 142) of specimens to species. Of these morphological variables, head depth and caudal-peduncle depth exhibit the most consistent differences between species. Third, the two species exhibit marked differences in morphology of the gonopodial tip [Fig. 5(b) and Table II]. Three PC axes were retained for analysis of the gonopodial tip (Table IV), and species significantly differ along only the first axis (PC1:  $F_{1,41.88} = 224.72$ ,  $P < 0.001$ ; PC2:  $F_{1,39.27} = 0.13$ ,  $P > 0.05$ ; PC3:  $F_{1,44.62} = 0.76$ ,  $P > 0.05$ ). This indicates that in a multivariate sense, gonopodia are highly distinctive among species. The morphology of the gonopodial tip of *G. quadruncus* differs, on average, from that of *G. affinis* in typically having fewer and shorter serrae on ray 4p, having a more distally positioned elbow on ray 4a, typically having a larger gap height between rays 4a and 4p distal to the elbow, having fewer spines on ray 3 and having a terminal hook at the distal tip of the gonopodium. Thus, the two species differ on average in many gonopodial characters and there is a considerable overlap in many of these individual traits, with two traits (elbow position and terminal hook) serving as the best diagnosable characters (Table II). Based on morphology of the gonopodial tip, DFA correctly assigned 99% (107 of 108) of specimens to species. Fourth, *G. quadruncus* usually possesses 32 (male) or 31–32 (female) vertebrae (including the urostylar half-centrum as a single element), while *G. affinis* typically possess 33 (male) or 32 (female) vertebrae (Rosa-Molinar *et al.*, 1998; R. B. Langerhans, unpubl. data).

*Gambusia quadruncus* also differs from *G. affinis* in colouration, having light-orange unpaired fins, generally more strongly blackened margins of caudal and anal (female) fins, caudal fins that are typically more strongly spotted, a generally stronger postanal streak and anal spots in females that are permanent, larger, darker and positioned more posteriorly (Table III). Of particular note is the difference in anal spot expression, which could influence reproductive isolation among the species as the spot has been implicated in guiding gonopodial orientation in male *Gambusia* (Peden, 1973b). While *G. affinis* typically lacks anal spots or exhibits cyclic expression of anal spots based on the ovarian cycle (Hubbs, 1959; Peden, 1973b), virtually all female specimens of *G. quadruncus* examined here possess distinctive anal spots [see Fig. 3(b), showing a darkened region just above anal fin rays 2–5], with the exception of specimens from collections in the relatively isolated, north-eastern Gulf of Mexico drainages (UMMZ 169634, UMMZ 184386, UMMZ 184389 and UMMZ 184404). It is unclear why these latter populations might lack expression of an otherwise distinctive characteristic of *G. quadruncus*.

#### Phylogenetic analyses

Analysis of mtDNA sequences yields strongly supported phylogenetic relationships [Fig. 6(a)]. Both ML and BI analyses provided strong support for reciprocal monophyly among *G. quadruncus* and its close relatives (*G. affinis* and *G. holbrooki*). Analysis of S7 sequences provides poor resolution regarding the relationships between *G. affinis* and *G. quadruncus*, although S7 results suggest a probable sister relationship between these two species, albeit with incomplete lineage sorting (Appendix III). For the combined dataset, ML and BI analyses generate consistent and strongly supported phylogenetic relationships, supporting the reciprocal monophyly of *G. quadruncus* and its close relatives [Fig. 6(b)]. Combined analysis of mtDNA and nDNA confirm a sister relationship between *G. affinis* and *G. quadruncus*, and indicates an apparent lack of gene flow between the species for a considerable amount of time, especially considering the inclusion of specimens for these two species collected from the same drainage (localities 1 and 4). Examination of pair-wise genetic distances among samples (corrected for the selected model of sequence evolution) indicates that levels of genetic divergence between *G. quadruncus* and *G. affinis* are generally more than twice as strong as intraspecific nucleotide variation within each species. For the samples examined here, pair-wise uncorrected per cent nucleotide differences between *G. quadruncus* and *G. affinis* (*p*-distance) is 0.995–1.493% for *cyt b*, 2.462–3.179% for ND2 and 0.393–0.916% for S7.

To provide a rough estimate of divergence time between the sister species *G. quadruncus* and *G. affinis*, the 95% c.i. of divergence time estimates of Hrbek *et al.* (2007) for a node within the genus *Gambusia* were used to construct a molecular clock for the regions of *cyt b* and ND2 examined here (the only overlapping gene regions between the two studies). Using sequence data from Hrbek *et al.* (2007), a molecular clock of 0.7–0.9% sequence divergence per million years was estimated for the *cyt b* fragment and 1.2–1.5% sequence divergence per million years for the ND2 fragment. Based on these molecular clock estimates, the two sister species appear to have diverged from one another *c.* 1.2–2.9 million years ago (1.2–2.3 million years ago using *cyt b* divergence of 1.0–1.6%; 1.6–2.9 million years ago using ND2 divergence of 2.5–3.5%).

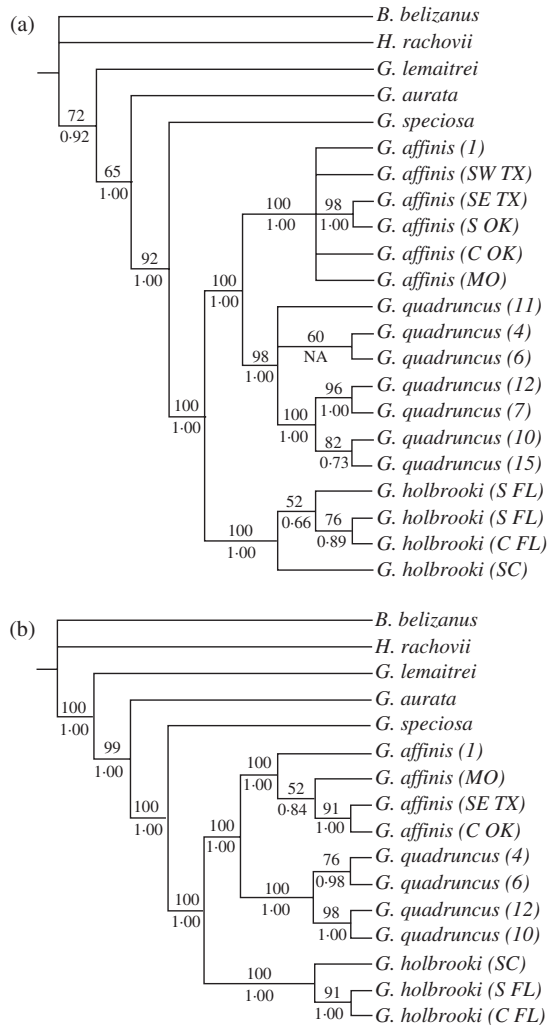


FIG. 6. Maximum likelihood phylogeny using (a) concatenated mtDNA and (b) a combined analysis of mtDNA and the nuclear encoded S7 intron. Numbers above and below branches indicate maximum likelihood bootstrap percentages and Bayesian inference posterior probabilities for each node, respectively. The geographical region of each sample of *Gambusia affinis*, *Gambusia quadruncus* and *Gambusia holbrooki* is given in parentheses, with numbers referring to site localities in east-central México depicted in Fig. 1.

### Distribution and habitat

*Gambusia quadruncus* is known from east-central México, within the following drainages: Río Guayalejo-Tamesí, Río Pánuco, Laguna de Tamiahua, Río Tigre, Río Carrizal and Río Soto La Marina. This range spans at least three states (Tamaulipas, Veracruz and San Luis Potosí) and encompasses over 25 000 km<sup>2</sup> (Fig. 1). In the southern extent of its range, *G. quadruncus* might be found in the extreme northern parts of the state of Hidalgo, but no such collections are known. The range of *G. quadruncus* appears restricted to the Gulf coastal plain, narrowly overlapping with the range of *G. affinis* in the Río Soto La Marina drainage in the vicinity of

Ciudad Victoria. Thus, the sister species may occur sympatrically; for instance, the two species are known from nearby collections within the same river (Río Corona) taken at different times (UMMZ 169611 from 1941 and CPUM 2676 from 2007). Morphological and molecular data examined for specimens of both species collected in the Río Soto La Marina drainage (sites 1–4 in Fig. 1) revealed no evidence for hybridization or clinal convergence in traits, suggesting the two species exhibit a strong degree of reproductive isolation within this potential region of sympatry. If the species indeed co-occur in this region, this small zone of sympatry might reflect secondary contact, as most of the species' ranges are separated by mountainous stretches. Specifically, the two species primarily occur on the western (*G. affinis*) and eastern (*G. quadruncus*) sides of the Tamaulipas Arch, the north-south chains of Sierra de San Carlos, Mesa de Solís and Sierra de Tamaulipas (= Sierra Dientes de Moreno); and the two species appear largely divided to the north (*G. affinis*) and south (*G. quadruncus*) by the Lleran Mesas, the lava-capped mesas near Llera de Canales, between the Sierra Madre Oriental and Sierra de Tamaulipas. Only in a small region in the west-central area of the Tamaulipas Arch might the two species coexist. The accuracy of these range estimates, as well as the precise north-eastern extent of the range of *G. quadruncus* await further study. Rauchenberger (1989) suggested that the southernmost occurrence of *G. affinis* was Tampico (c. 22.2° N), although she noted that further examination would be necessary to confirm that these fish were indeed *G. affinis* and not a close relative, such as *G. speciosa*. Obregón-Barboza *et al.* (1994) indicated that the southern limit of *G. affinis* was at least as far south as Laguna de Tamiahua (c. 21.3° N). Indeed, the collection from Laguna de Tamiahua examined here had been catalogued as *G. affinis* in the University of Michigan Museum of Zoology since 1979. It is found here that these southern reports of *G. affinis* are *G. quadruncus*, with the actual southernmost known occurrence for *G. affinis* (and *G. speciosa*) being around Ciudad Victoria (c. 23.7° N).

*Gambusia quadruncus* inhabits a wide range of aquatic habitats, being observed in ponds, drainage ditches, lakes, wetlands, brackish lagoons, large rivers and swiftly flowing spring-fed streams. The species is often sympatric with other poeciliids, including *G. aurata*, *G. panuco*, *G. vittata*, *Poecilia formosa* (Girard 1859), *Poecilia latipunctata* Meek 1904, *Poecilia mexicana* Steindachner 1863 and *Xiphophorus variatus* (Meek 1904). In the type locality, all four of these species of *Gambusia* were observed. Miller & Minckley (1970) previously noted the occurrence of four sympatric species of *Gambusia*. Like Miller & Minckley (1970), strong habitat segregation among the four congeners was observed in the type locality. *Gambusia quadruncus* was collected in still water on the margins of vegetation beds, *G. aurata* was found within thick vegetation, *G. vittata* was collected in areas with higher water-flow velocities and *G. panuco* was found in slow-velocity, open-water sections.

#### Etymology

The specific name is from the Latin (*quad*: four and *uncus*: hook) in reference to the four hooked elements at the distal tip of the gonopodium. The common name, llanos mosquitofish, refers to the region inhabited by the species, the Mexican gulf coastal plain.



**KEY TO SPECIES OF *GAMBUSIA* IN *AFFINIS* SPECIES GROUP IN  
EAST-CENTRAL MÉXICO**

- 1A. Body colour golden to orange; gonopodial ray 3 foreshortened, not reaching distal tip; rounded hook at distal end of gonopodial ray 5a. Atlantic Slope, Río Guayalejo-Tamesí system ..... *Gambusia aurata*
- 1B. Body colour not golden or orange; gonopodial ray 3 extending to distal tip; hook at distal end of gonopodial ray 5a pointed proximally ..... 2
- 2A. Posterior surfaces of gonopodial ray 3 segments denticulate proximal to spines. Not native to México, but probably introduced. .... *Gambusia holbrooki*
- 2B. Posterior surfaces of gonopodial ray 3 segments smooth proximal to spines . . . . . 3
- 3A. Gonopodial ray 4p arching anteriorly at tip towards ray 4a (but generally not touching or interconnected); elbow on ray 4a comprising four or more fused elements. Atlantic Slope, Río Bravo drainage, southwards into Río Soto la Marina ..... *Gambusia speciosa*
- 3B. Gonopodial ray 4p almost straight or curved posteriorly at tip; elbow on ray 4a usually comprising two or three fused elements. .... 4
- 4A. Distal tip of gonopodium acuminate; serrae on gonopodial ray 4p extending distal to elbow. Atlantic Slope, Río Bravo drainage, southwards into Río Soto la Marina ..... *Gambusia affinis*
- 4B. Hook at distal tip of gonopodium formed by ends of rays 4a, 4p or both; serrae on gonopodial ray 4p often proximal to elbow. Atlantic Slope, Río Soto la Marina, Río Guayalejo-Tamesí, Río Pánuco, Laguna de Tamiahua, Río Tigre, Río Carrizal drainages ..... *Gambusia quadruncus*

**DISCUSSION**

The description of *G. quadruncus* adds to an already diverse, monophyletic genus of livebearing fishes. Moreover, its description highlights the hotspot of diversity of *Gambusia* in east-central México. This region exhibits a remarkably high level of endemism for freshwater fishes (Miller *et al.*, 2005; Abell *et al.*, 2008), and its contribution to biodiversity is evident in the genus *Gambusia*: no other biogeographical region harbours as many valid species of *Gambusia*, nor as many putative undescribed species. It has been previously hypothesized that orogenic events since the Miocene have resulted in the isolation and subsequent speciation of freshwater fishes in this region (Miller & Smith, 1986; Schönhuth *et al.*, 2008). The origin of *G. quadruncus* appears consistent with this hypothesis: volcanic activity in the Tamaulipas Arch and Lleran Mesas during the Pliocene (de Cserna, 1960; Robin & Tournon, 1978; Camacho-Angulo, 1993; Aranda-Gómez *et al.*, 2002, 2007) apparently created a vicariant event, resulting in the isolation (or partial isolation) of a once continuously distributed lineage of *Gambusia*. Molecular estimates of divergence time between *G. affinis* and *G. quadruncus* (1.2–2.9 million years ago) fall

within the later stages of this volcanic activity (1.8–5.0 million years ago), and suggest a causal role for Pliocene orogenesis in the speciation process.

Orogenic events associated with the Tamaulipas Arch and Sierra Madre Oriental not only provide vicariant events, but can also serve as barriers preventing fishes that arrived after the events from traversing the mountains. Indeed this region serves as a range limit for a number of freshwater fishes, including many poeciliids, e.g. *G. aurata*, *G. speciosa*, *G. vittata*, *P. latipunctata*, *X. variatus* and *Xiphophorus xiphidium* (Gordon 1932). Combined with the importance of the Trans-Mexican Volcanic Belt and the Los Tuxtlas volcanoes in areas south of the study area examined here (Miller & Smith, 1986; Obregón-Barboza *et al.*, 1994; Contreras-Balderas *et al.*, 1996; Mateos *et al.*, 2002; Hulsey *et al.*, 2004; Kallman & Kazianis, 2006; McEachran & Dewitt, 2008), this suggests a major role for late Miocene and Pliocene orogenesis in the complex biogeography of freshwater fishes in east-central México.

*Gambusia quadruncus* differs from its closest relative, *G. affinis*, in several characters that could prevent significant interbreeding when sympatric. That is, the two species differ in  $L_S$ , body shape, gonopodium length, gonopodial-tip morphology, unpaired fin colouration and female anal spot expression; all of these traits have been implicated in mating activities that could influence reproductive isolation in poeciliids (Peden, 1972a, b, 1973b; Hughes, 1985; McPeck, 1992; Houde, 1997; Pilastro *et al.*, 1997; Langerhans *et al.*, 2005, 2007). Thus, in addition to molecular evidence suggesting restricted gene flow between the species, there are numerous morphological lines of evidence that suggest the likelihood of reproductive isolation.

Many species of *Gambusia* face threats of extinction due to human-induced environmental changes, with nine species currently listed as threatened in the U.S.A. and México, and three species apparently having gone extinct within the last 40 years (Peden, 1973a; Johnson & Hubbs, 1989; Edwards *et al.*, 2002; Hubbs *et al.*, 2002a, b, c; Tobler & Plath, 2009; Langerhans, 2011). Fortunately, *G. quadruncus* does not have a narrowly restricted range like some species of *Gambusia*, and is unlikely to face serious threats of extinction in the near future. Accordingly, the species should be classified as Least Concern (LC) according to the IUCN Red List categories (IUCN, 2011). Rather than being threatened with extinction, this species may be more likely to pose a threat of its own *via* invasiveness in non-native regions if introduced to new regions. That is, *G. quadruncus* is closely related to two highly invasive species (*G. affinis* and *G. holbrooki*) that have been introduced throughout the world. Because of this phylogenetic relationship, *G. quadruncus* might share traits that confer invasiveness in non-native regions, although this is currently not known.

Inhabiting a region with a relatively well-studied ichthyofauna, and exhibiting a range covering >25 000 km<sup>2</sup>, how has *G. quadruncus* gone without description for so long? The species does share many superficial similarities with *G. affinis*, and has a range that abuts, or narrowly overlaps with the range of *G. affinis*. Thus, it is understandable that most ichthyologists previously identified *G. quadruncus* as *G. affinis*. The species, however, was noted as a peculiarity prior to this description. According to notes found attached or inside collection jars at UMMZ, *G. quadruncus* specimens were sometimes recognized as distinct from *G. affinis* by particular observers between the 1930s and 1970s. Specifically, notes by C. L. Hubbs, R. R. Miller and B. L. H. Brett (unpubl. data) indicated that the specimens were believed to represent a new subspecies (or perhaps multiple subspecies) of *G. affinis*. These particular

specimens, identified here as *G. quadruncus*, were collected from the Río Pánuco and Río Soto La Marina drainages (UMMZ catalogue numbers: 97553–97556, 164735, 169640 and 209492). Moreover, M. Rauchenberger noted in her key to Mexican Poeciliidae in Miller *et al.* (2005) that the status of southern populations of *G. affinis* were unclear (*i.e.* populations south of the Río Bravo drainage). According to a note inside the collection jar of UMMZ 169640, R. R. Miller noted in 1973 that these specimens should be re-examined at a later date for taxonomic clarification. Through an independent route, this suggestion was taken up here with the description of the new species *G. quadruncus*.

## COMPARATIVE MATERIALS EXAMINED

### MORPHOLOGICAL SPECIMENS

During the course of this study, numerous collections were examined for measurement and identification. In the collections maintained by the research laboratory of R. B. Langerhans (Langerhans Laboratory Specimen and Tissue Collection, LLSTC), the following was examined: 34 collections of *G. affinis* (LLSTC C00007, C00009, C00088, C000091–000099, C00101–00103, C00135–00136, C00143, C00252–256 and C00327–00337), three collections of *G. aurata* (C00041, C00131 and C00316), one collection of *G. lemaitrei* (C00162), four collections of *G. panuco* (C00041, C00131, C00313 and C00315), two collections of *G. speciosa* (C00037 and C00038) and four collections of *G. vittata* (C00041, C00131, C00314 and C00316). At UMMZ, the following collections were identified as *G. quadruncus*: 97553, 97554, 97555, 97556, 164735, 169634, 169640, 170944, 180042, 180060, 181292, 181791, 184380, 184386, 184389, 184404, 186499, 192874, 192879, 192886, 196898, 196907, 209492 and 209510; and the following collections were identified as *G. affinis*: 162148, 169611, 169624 and 192910. At CPUM, one collection of *G. regani* (4406) was examined, five collections were identified as *G. quadruncus* (2672–2676) and one collection was identified as *G. affinis* (2715).

### MOLECULAR SPECIMENS

For each species, voucher catalogue number, collection locality and GenBank accession numbers are listed. Voucher specimens/tissues were deposited in the Colección de Peces de la Universidad Michoacana de San Nicolás de Hidalgo (CPUM), and the Langerhans Laboratory Specimen and Tissue Collection (LLSTC).

#### *Belonesox belizanus*

LLSTC 04587, Amalgres, México, HM443900, HM443919, HM443938.

#### *Gambusia affinis*

CPUM 2715, Mainero, México, HM443902, HM443921, HM443940; LLSTC 04577, Maverick County, Texas, HM443905, HM443922; LLSTC 04578, Brazos County, Texas, HM443906, HM443923, HM443941; LLSTC 04585, Marshall County, Oklahoma, HM443907, HM443924; LLSTC 04579, Cleveland County,

Oklahoma, HM443905, HM443925, JN128635; LLSTC 04580, Shannon County, Missouri, HM443903, HM443926, HM443942.

*Gambusia holbrooki*

LLSTC 04581, Monroe County, Florida, HM443915, HM443934; LLSTC 04582, Miami-Dade County, Florida, HM443916, HM443935, HM443948; LLSTC 04583, Manatee County, Florida, HM443917, HM443936, HM443947; LLSTC 04586, Richland/Lexington Counties, South Carolina, HM443918, HM443937, JN128636.

*Gambusia aurata*

LLSTC 11425, El Limón, México, JF437627, JF437630, JF637633.

*Gambusia lemaitrei*

LLSTC 11427, Bolivar, Colombia, JF437626, JF437629, JF437632.

*Gambusia quadruncus*

CPUM 2672, Forlon, México, HM443913, HM443929; CPUM 2673, Aldama, México, HM443914, HM443933; LLSTC 04571, Ciudad Victoria, México, HM443-908, HM443927, HM443943; LLSTC 04572, Ciudad Mante, México, HM443911, HM443928, HM443944; LLSTC 04573, El Limón, México, HM443909, HM443930, HM443945; LLSTC 04574, Llera, México, HM443912, HM443932, HM443946; LLSTC 04576, Tampico, México, HM443910, HM443931.

*Gambusia speciosa*

LLSTC 11426, Val Verde County, Texas, JF437628, JF437631, JF637634.

*Heterophallus rachovii*

LLSTC 04584, Amalgres, México, HM443901, HM443920, HM443939.

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APPENDIX I. Sample sizes of adult males examined for standard length ( $L_S$ ), body and fin morphology and morphology of the gonopodial tip

Species	Location	Collection	$L_S$ $n$	Body and fin morphology $N$	Gonopodial tip $n$	Fig. 1 site number
<i>Gambusia affinis</i>	Shannon County, Missouri	LLSTC C00092	1	1	1	1
<i>G. affinis</i>	Cleveland County, Oklahoma	LLSTC C00328	1	1	1	1
<i>G. affinis</i>	McClain County, Oklahoma	LLSTC C00327	3	3	3	3
<i>G. affinis</i>	Marshall County, Oklahoma	LLSTC C00336–00337	20	10	10	10
<i>G. affinis</i>	Brazos County, Texas	LLSTC C00330–00335	71	51	17	17
<i>G. affinis</i>	Hays County, Texas	LLSTC C00329	2	2	2	2
<i>G. affinis</i>	Near Ciudad Victoria, Tamaulipas, México	UMMZ 162148	18	10	10	2
<i>Gambusia</i>	Near Jimenez, Tamaulipas,	UMMZ 169634	18	5	5	3
<i>quadruncus</i>	México					
<i>G. quadruncus</i>	Near Lavaderos, Tamaulipas, México	UMMZ 184386	18	5	5	5
<i>G. quadruncus</i>	Near Llera, Tamaulipas, México	TNHC 45835	2	2	2	6
<i>G. quadruncus</i>	Aldama, Tamaulipas, México	UMMZ 184404	18	5	5	8
<i>G. quadruncus</i>	Near El Limón, Tamaulipas, México	UMMZ 248855, 248857, 181292	27	21	21	9, 10
<i>G. quadruncus</i>	Ciudad Mante, Tamaulipas, México	TNHC 45834, UMMZ 186499	12	12	12	12, 13
<i>G. quadruncus</i>	Altamira, Tamaulipas, México	UMMZ 192874	2	2	2	14
<i>G. quadruncus</i>	Tampico, Tamaulipas, México	UMMZ 209492	11	5	5	16
<i>G. quadruncus</i>	Near Ebano, San Luis Potosí, México	UMMZ 170944	1	1	1	17
<i>G. quadruncus</i>	El Pujal, San Luis Potosí, México	UMMZ 196898	1	1	1	18
<i>G. quadruncus</i>	Near El Higo, Veracruz, México	UMMZ 97555	4	4	4	19
<i>G. quadruncus</i>	Tamiahua, Veracruz, México	UMMZ 209510	1	1	1	20



APPENDIX II. Population sources for molecular data in the examination of genetic distinctiveness of *Gambusia quadruncus*

Species	Location	Collector(s)	Genes sequenced	Fig. 1 site	number
<i>Gambusia affinis</i>	Shannon County, Missouri	R. B. Langerhans	ND2, cyt b, S7		
<i>G. affinis</i>	Cleveland County, Oklahoma	P. Reneau	ND2, cyt b, S7		
<i>G. affinis</i>	Marshall County, Oklahoma	P. Reneau	ND2, cyt b		
<i>G. affinis</i>	Brazos County, Texas	R. B. Langerhans	ND2, cyt b, S7		
<i>G. affinis</i>	Maverick County, Texas	E. Marsh-Matthews	ND2, cyt b		
<i>G. affinis</i>	Near Mainero, Tamaulipas, México	O. Domínguez-Domínguez, R. Pérez-Rodríguez	ND2, cyt b, S7		1
<i>Gambusia quadruncus</i>	Near Ciudad Victoria, Tamaulipas, México	O. Domínguez-Domínguez, R. Pérez-Rodríguez	ND2, cyt b, S7		4
<i>G. quadruncus</i>	Near Llera, Tamaulipas, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b, S7		6
<i>G. quadruncus</i>	Near Fortlon, Tamaulipas, México	O. Domínguez-Domínguez, R. Pérez-Rodríguez	ND2, cyt b		7
<i>G. quadruncus</i>	Near El Limon, Tamaulipas, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b, S7		10
<i>G. quadruncus</i>	Near Aldama, Tamaulipas, México	O. Domínguez-Domínguez, R. Pérez-Rodríguez	ND2, cyt b		11
<i>G. quadruncus</i>	Ciudad Mante, Tamaulipas, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b, S7		12
<i>G. quadruncus</i>	Tampico, Tamaulipas, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b		15
<i>Gambusia holbrooki</i>	Richland/Lexington Counties, South Carolina	C. Rinehart	ND2, cyt b, S7		
<i>G. holbrooki</i>	Manatee County, Florida	R. B. Langerhans, C. Ruehl	ND2, cyt b, S7		
<i>G. holbrooki</i>	Miami-Dade County, Florida	R. B. Langerhans, C. Ruehl	ND2, cyt b, S7		
<i>G. holbrooki</i>	Monroe County, Florida	R. B. Langerhans, C. Ruehl	ND2, cyt b		
<i>Gambusia aurata</i>	Near El Limon, Tamaulipas, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b, S7		
<i>Gambusia lemaitrei</i>	Lago Totumo, Bolívar, Colombia	M. Torres-Mejía	ND2, cyt b, S7		
<i>Gambusia speciosa</i>	Val Verde County, Texas	P. Reneau	ND2, cyt b, S7		
<i>Heterophallus rachovii</i>	Near Amalgres, Veracruz, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b, S7		
<i>Betonesox belizanus</i>	Near Amalgres, Veracruz, México	R. B. Langerhans, T. J. DeWitt, D. García-Bedoya	ND2, cyt b, S7		

APPENDIX III. Maximum likelihood phylogeny based on the nuclear encoded S7 intron. Numbers above and below branches indicate maximum likelihood bootstrap percentages and Bayesian inference posterior probabilities for each node, respectively. The geographical region of each sample of *Gambusia affinis*, *Gambusia quadruncus* and *Gambusia holbrooki* is given in parentheses, with numbers referring to site localities in east-central México depicted in Fig. 1.

