



A new species of *Isthmura* (Caudata: Plethodontidae) from the montane cloud forest of central Veracruz, Mexico

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Abstract

We describe a new plethodontid salamander species of the genus *Isthmura*, known only from one locality in the mountainous region of central Veracruz, Mexico. Like its congeners, *Isthmura corrugata* **sp. nov.** has a large and robust body, but it is easily distinguished from the other species in the genus by the absence of any spot or mark on the dorsum (except by dull reddish brown coloration on eyelids) and by extremely well-marked costal grooves separated by very pronounced costal folds. Based on an mtDNA phylogeny, the new species is most closely related to the geographically distant *I. boneti* and *I. maxima* but occurs very near *I. naucampatepetl* and *I. gigantea* on the eastern slope of Cofre de Perote, Veracruz. The region where *I. corrugata* occurs contains a high number of plethodontid salamander species and is threatened by human activity.

Key words: Amphibians, *Isthmura corrugata* **sp. nov.**, morphology, Neotropics, phylogeny, salamander

Introduction

The Neotropical salamander genus *Pseudoeurycea* was recently split into three genera: *Aquiloerycea*, *Isthmura*, and *Pseudoeurycea* (Rovito *et al.* 2015). Salamanders of the genus *Isthmura*, formerly recognized as the *Pseudoeurycea bellii* complex (Parra-Olea *et al.* 2005), are the largest terrestrial plethodontids in the Neotropical region (Rovito *et al.* 2015). They are black, robust, large-bodied animals commonly with red, orange, or pink dorsal coloration on a black background and slight interdigital webbing with a well-developed fifth toe. The genus *Isthmura* currently contains six species: *I. bellii*, *I. boneti*, *I. gigantea*, *I. maxima*, *I. naucampatepetl*, and *I. sierraoccidentalis*. All species occur exclusively in Mexico, although Bezy *et al.* (2004) discuss the remote possibility that *I. sierraoccidentalis* might occur in Arizona. Most species inhabit montane forests above 2,000 m elevation, excluding *I. gigantea* (1,000–2,000 m) and *I. maxima* (ca 750–2,300 m.).

We have been assessing the current status of amphibians in Veracruz, Mexico, a state with both high diversity and high levels of threat. We have searched both in forest and in habitat modified by human activity over the course of several years. During fieldwork in 2014 in a cloud forest of the mountainous central region of Veracruz, we found two stout, black salamanders without spots or marks on the dorsum (except by dull reddish brown coloration on eyelids) and a corrugated body, unlike any known species. Here, we describe a new species of *Isthmura* from Veracruz based on both morphological and molecular evidence and discuss its phylogenetic relationships. The new salamander species seems to be rare based on the low number of individuals recorded, in spite of searches at type locality and surroundings during several years.

Material and methods

Morphological analysis. We followed the standard format for *Pseudoeurycea* species descriptions proposed by Lynch & Wake (1989), previously used for the *bellii* group (Parra-Olea *et al.* 2005), which now constitutes the genus *Isthmura*. We measured 27 traits from the holotype and paratype: standard length (distance from snout to posterior end of vent; SL); head width; snout to gular fold (head length); head depth; eyelid length; eyelid width; anterior rim of orbit to snout; eye diameter; interorbital distance; distance between vomerine teeth and parasphenoid tooth patch; snout to forelimb distance; internarial distance; intercanthal distance; nostril diameter; snout projection beyond mandible; snout to anterior angle of vent; axilla-groin distance; tail length; tail width at base; tail depth at base; forelimb length (to tip of longest digit); hind limb length (to tip of longest toe); width of hand; width of foot; length of the longest (third) toe; mental gland width; mental gland length. We also counted the total numbers of premaxillary, maxillary, and vomerine teeth. Larger measurements were made using a digital caliper (Mitutoyo; 0.01mm resolution), while smaller measurements such as foot width, toes length, and head dimensions were made using a stereoscopic microscope (model Leica S6D). We followed Köhler (2012) to describe coloration in life and in alcohol of the holotype and paratype (color codes are included in parenthesis). Skeletal characters were assessed using X-rays.

DNA extraction and sequencing. We extracted DNA from ethanol-preserved tissue samples of two individuals of the new species using a Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). We amplified the large subunit ribosomal RNA (*16S*) and cytochrome *b* (*cytb*) mitochondrial genes using primers 16Sar/16Sbr (Palumbi *et al.* 1991) for *16S* and MVZ15/MVZ16 (Moritz *et al.* 1992) for *cytb*. PCR consisted of an initial denaturation step at 94 °C for 2 min, followed by 38 cycles of 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min, with a final annealing step at 72 °C for 7 min. PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA), sequenced with BigDye 3.1 terminator chemistry, and sequenced on an ABI 3710 sequencer (Applied Biosystems, Foster City, CA). We edited sequences using Geneious v.5.6 (www.geneious.com, Kearse *et al.* 2012).

Phylogenetic analyses. We obtained sequences of *16S* and *cytb* for members of the genera *Aquiloerycea*, *Isthmura*, *Ixalotriton*, and *Pseudoeurycea* from Genbank; these four genera form a clade in the mitochondrial phylogeny of Rovito *et al.* (2015). Sequences were aligned using MUSCLE v.3.8 (Edgar 2004) with default parameters and trimmed to the point where a majority of taxa had sequence data. After preliminary analyses with species from all four genera indicated a close relationship between the new species and the genera *Isthmura* and *Aquiloerycea*, we conducted phylogenetic analyses using only members of those two genera in order to reduce ambiguously aligned regions of the *16S* gene between highly divergent species. We used *Pseudoeurycea rex* as outgroup for all phylogenetic analyses. Voucher information and Genbank numbers for species of *Isthmura* and *Aquiloerycea* used in phylogenetic analyses are given in Table 1.

We partitioned the sequence data by gene, and further partitioned *cytb* by codon position. We used the program PartitionFinder v1.0 (Lanfear *et al.* 2012) to determine the best partitioning strategy and nucleotide substitution model for each partition, using the Bayesian Information Criterion (BIC) to choose between partitioning schemes. The following partitioning scheme and nucleotide substitution models were selected: *16S*, *cytb* codon positions 1 and 2: HKY+I; *cytb* codon position 3: HKY. We used both Maximum Likelihood (ML) and Bayesian methods to estimate a phylogeny, and all analyses were run on the CIPRES Science Gateway (Miller *et al.* 2010). Maximum likelihood analysis was done using the program RAxML v8.0.24 (Stamatakis 2006), with the partitioning strategy given above and a GTR+G model for each partition (RAxML does not implement simpler models) and one thousand bootstrap replicates to assess support. Bayesian analyses were performed using MrBayes 3.2 (Ronquist *et al.* 2012). Two separate analyses were run for 20,000,000 generations, sampled every 1,000 generations, and four chains per run with default heating parameters. The first 5,000 samples were discarded as burn-in.

Results

The combined *16S* and *cytb* mtDNA gene tree (Fig. 1) has two clades, corresponding to *Aquiloerycea* and *Isthmura*, each of which is monophyletic with high support (ML bootstrap [BS] >80, Bayesian posterior probability [PP]= 1.0). The two samples from the new Veracruz species were estimated to be part of the *Isthmura* clade, as the

sister taxon of (*Isthmura boneti* + *Isthmura maxima*) with high support in the Bayesian analysis (PP= 1.0), but with only weak support in the ML analysis (BS= 0.62). Within *Isthmura*, the new Veracruz species has a minimum Tamura-Nei distance of 0.014 to *I. maxima* and a maximum distance of 0.046 to *I. sierraoccidentalis* for *16S*, and a minimum distance of 0.10 to *I. boneti* and a maximum distance of 0.16 to *I. sierraoccidentalis*. In combination with the phylogenetic results, morphological differences (see below) of the specimens from Coatepec, Veracruz provide us enough evidence to recognize this salamander as separate evolutionary unit of *Isthmura*, and we describe it as new species.

TABLE 1. Catalog number, locality information, and GenBank accession numbers for *16S* and *cytb* sequences used in phylogenetic analyses.

Species	Voucher number	Locality	<i>16S</i> GenBank number	<i>cytb</i> Genbank number
<i>Aquiloerycea cafetalera</i>	IBH 14341	México: Veracruz: Rancho dos Puentes, Huatusco.	HM365064	-
<i>Aquiloerycea cephalica</i>	IBH 22603	México: Puebla: km 35, Apizaco-Chignahuapan.	KP886863	KP900066
<i>Aquiloerycea galeanae</i>	IBH 24595	México: Nuevo León: Puerto de Cieneguillas.	KP886847	KP886904
<i>Aquiloerycea quetzalanensis</i>	IBH 14208	México: Puebla: Xocoyolo.	HM365058	-
<i>Aquiloerycea scandens</i>	MVZ 173302	México: Tamaulipas: Rancho del Cielo.	AF451243	-
<i>Isthmura bellii</i>	MVZ 143795	México: Jalisco: 13.8 mi W of Ciudad Guzmán, N slope Nevado de Colima.	AY864691	AY864700
<i>Isthmura boneti</i>	MVZ 137857	México: Oaxaca: 3.1 mi W Cerro Machín on road to Comaltepec.	AY864712	AY864688
<i>Isthmura corrugata</i>	CARIE 0943	México: Veracruz: 9 km NW of Coatepec.	MF037230	MF037232
<i>Isthmura corrugata</i>	CARIE 0960	México: Veracruz: 9 km NW of Coatepec.	MF037231	MF037233
<i>Isthmura gigantea</i>	GP0177	México: Veracruz: La Joya.	AF451219	AF451198
<i>Isthmura maxima</i>	MVZ 146785	México: Oaxaca: 19.5 km NE of Putla de Guerrero.	AY862154	AY864684
<i>Isthmura naucampatepetl</i>	MVZ 173436	México: Veracruz: road to Las Lajas Microwave Station, 15 km S (by road) Highway 140.	-	AY864683
<i>Isthmura sierraoccidentalis</i>	IBH 24632	México: Chihuahua: 6 km W Ocampo.	KP886845	KP900049
<i>Pseudoeurycea rex</i>	MVZ 263590	Guatemala: San Marcos: 3 km E Flores de Ixchiguan.	KP886852	KP900056

***Isthmura corrugata* sp. nov.**

Suggested English name: Corrugated Salamander.

Suggested Spanish name: Salamandra corrugada.

(Figures 2, 3)

Holotype. CARIE 0943 (Colección de Anfibios y Reptiles del Instituto de Ecología, A.C.), an adult male collected 9 km NW of Coatepec, Veracruz, México (19° 29'N, 97° 02'W), 2,106 m elevation, collected by A. Sandoval-Comte (original field number ASC 200) at 22:15 h on 28 April 2014 (Fig. 2A–2F).

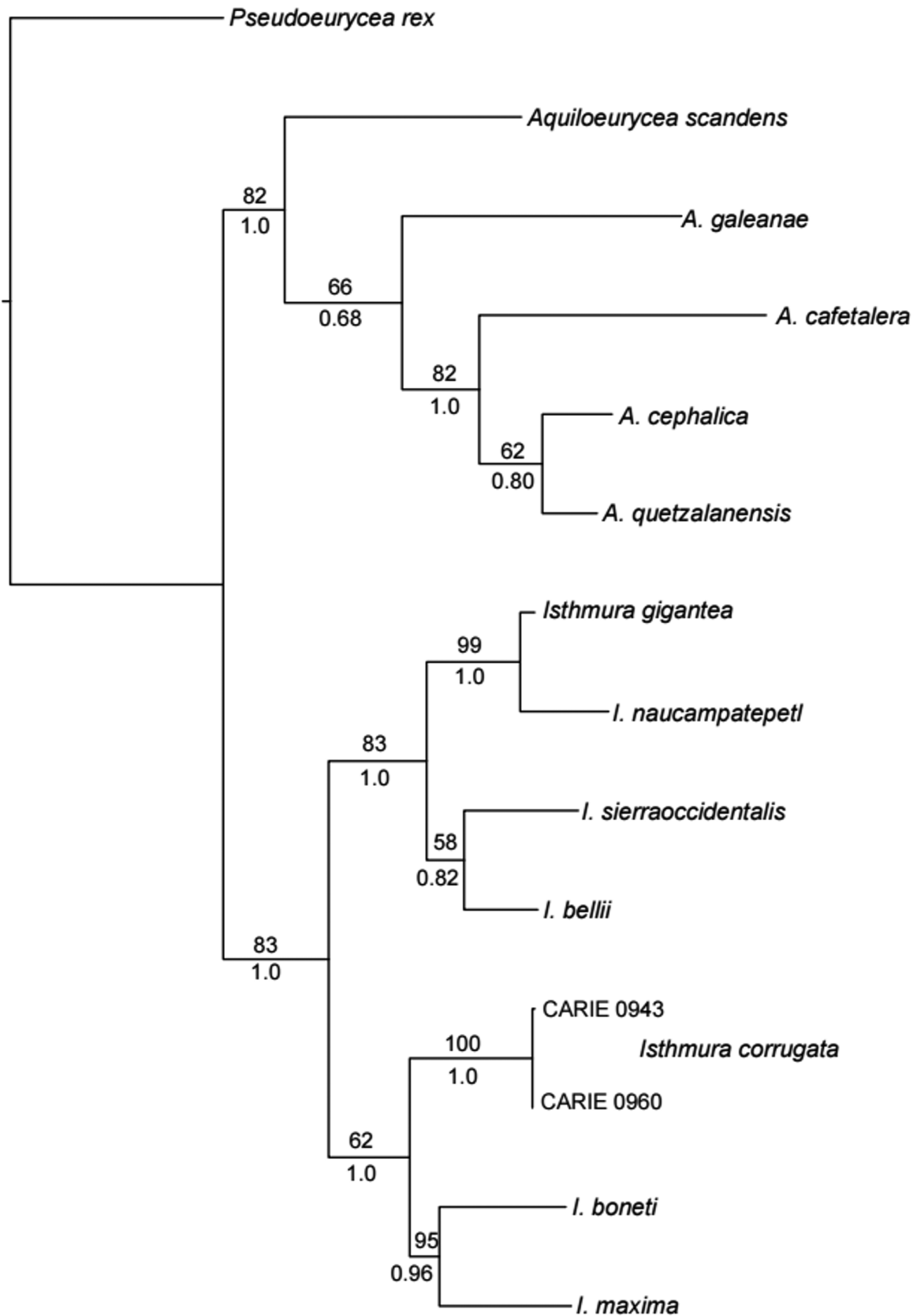


FIGURE 1. The combined 16S and cyt b mtDNA gene tree of *Aquiloeurycea* and *Isthmura* from maximum likelihood analysis. Numbers above branches correspond to bootstrap proportions, and numbers below branches correspond to posterior probabilities from Bayesian analysis. Branch lengths are in estimated number of substitutions per site.

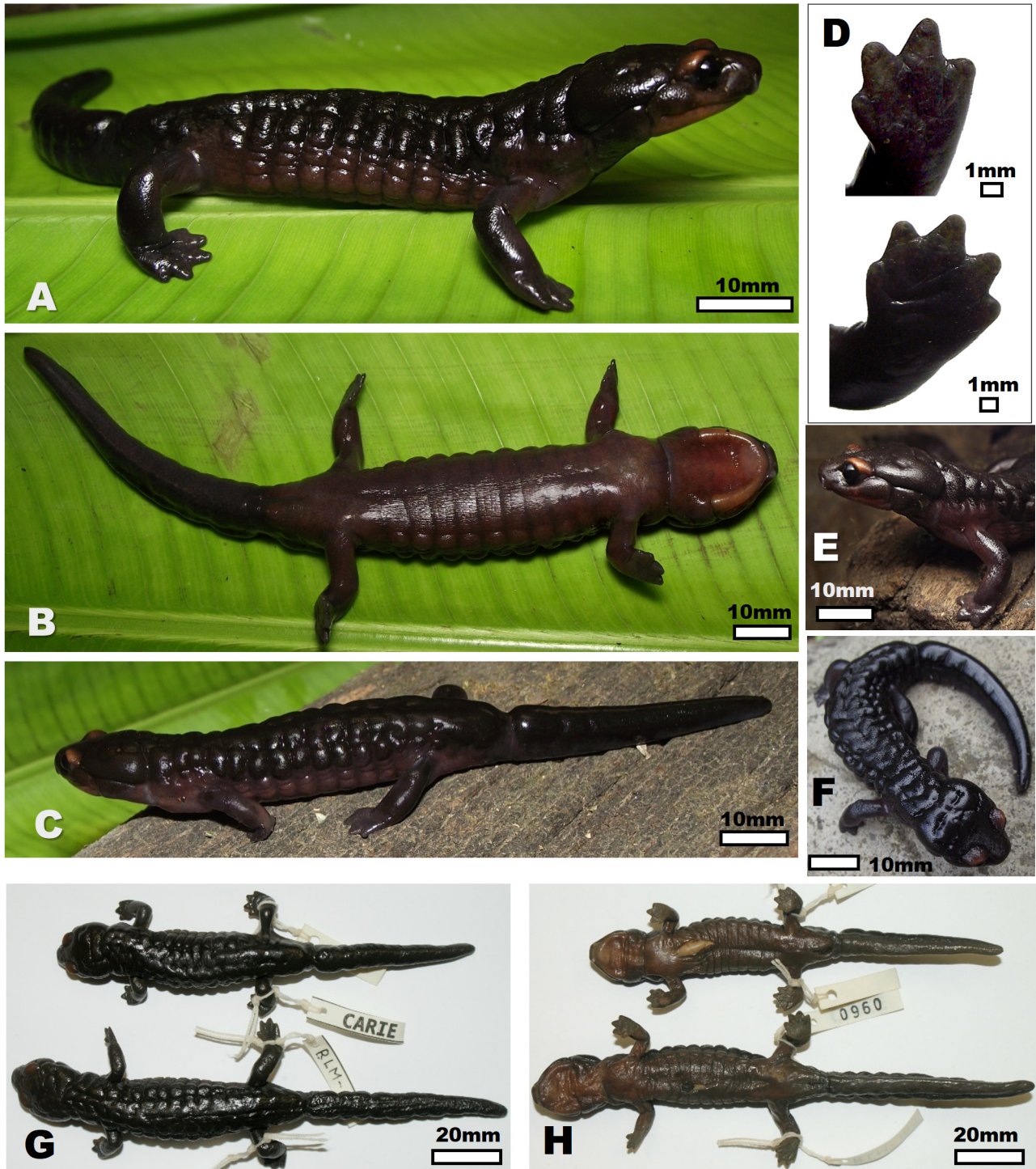


FIGURE 2. Holotype of *Isthmura corrugata*. A) Lateral and B) Ventral view. C) Coloration and form of costal grooves. D) Left hand (up) and left foot (down). E) Details of the head. F) Dorsal view. (G–H) Holotype (above) and paratype (below) dorsal and ventral coloration in preservation. All photographs by A. Sandoval-Comte.

Paratype. CARIE 0960, an adult male, same locality data as the holotype, collected by R. Luría-Manzano at 15:18 h on 4 August 2014 (Fig. 2G–2H).

Diagnosis. This is a plethodontid salamander of the genus *Isthmura*, based on its large size, robust body, and presence of a sublingual fold as well as on mtDNA sequence data. Differs from all other bolitoglossines except *Pseudoeurycea papenfussi* by its extremely well-defined and bulging costal folds, giving it a segmented appearance that extends even across the venter. Distinguished from all species of *Aquiloerycea* by its larger size

(*A. scandens* maximum SL 71 mm) and from similarly sized species of *Bolitoglossa* by the presence of a sublingual fold. Differs from large, robust species of *Pseudoeurycea* such as *P. papenfussi* and *P. smithi* by having more substantial foot webbing and triangularly shaped toes. It is the second smallest species of *Isthmura* (maximum SL 85.3 mm), only slightly larger than *I. naucampatepetl* (maximum SL 82.9 mm); all other species in the genus are substantially larger (maximum SL in: *I. sierraoccidentalis* 88.3 mm; *I. maxima* 128.1 mm; *I. bellii* 146 mm; *I. boneti* 107 mm; *I. gigantea* 161 mm; Parra-Olea *et al.* 2005; Raffaelli 2007). No females of *I. corrugata* have been found, so the maximum size of this species is likely greater than 85.3 mm SL because female *Isthmura* tend to be larger than males.

Members of the genus *Isthmura* have been characterized by the presence of spots or marks on a solid black dorsal background. The new species, however, is easily distinguished from all other members of the genus by the absence of any spot or mark on the dorsum, which instead is uniformly black, except by dull reddish brown coloration on eyelids. All other species have a series of chevrons (*I. boneti* and *I. gigantea*), half-chevrons (*I. bellii* and *I. maxima*), or small marks (*I. naucampatepetl*) along the dorsum, that are red-orange in all species except in *I. naucampatepetl*, in which they are pink (Parra-Olea *et al.* 2001; Parra-Olea *et al.* 2005). *Isthmura sierraoccidentalis* has a predominantly black dorsum, but all known individuals have some degree of reduced red-orange dorsal spotting (Lowe *et al.* 1968; Rovito *et al.* 2015). *Isthmura corrugata* is further distinguished by contrasting coloration between the blackish brown dorsal and vinaceous brown ventral background color, whereas all other species of the genus have a uniformly black dorsal and ventral background coloration. In addition, this species has a body marked by deep grooves and an unusual coloring above the eyes (dull reddish brown). Some body proportions also distinguish the new species from its congeners (Fig. 2). It has a relatively larger head (mean HL/SL= 0.23 versus 0.15–0.18 in the other species) and a relatively shorter tail (mean TL/SL= 0.69 versus 0.77–0.98 in the other species).

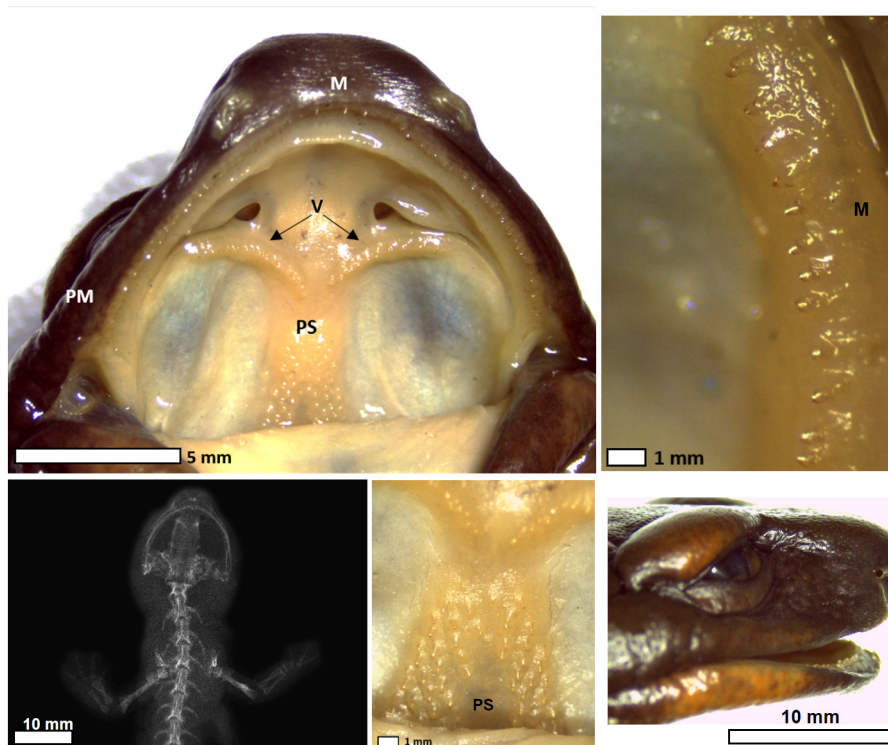


FIGURE 3. Osteological details of head and teeth detail of paratype of *Isthmura corrugata*: V= vomerine teeth, M= maxillary teeth and, PM= premaxillary teeth, PS= parasphenoid.

Description of holotype. Among the large salamanders of the genus *Isthmura*, this is a robust but relatively small species (maximum SL= 85.3 mm). Its body has a very muscular appearance. It has a prominent, broad (HW/SL= 0.22) and large head (HL/SL= 0.23) with very substantial jaw musculature. Deep postorbital groove extends from eye to end of jaw and continues to gular region. Snout is rounded and neck region is defined by deep groove behind head, slightly narrower than head. Parotoid glands are well developed and a large oval mental gland (mental

gland width/HW= 0.39) was present in both specimens. Eyes are protuberant. Maxillary teeth 62 (31/31), premaxillary teeth 6, enlarged compared to maxillary teeth; vomerine teeth 46, arranged in two arcs (22/24) extending beyond outer margin of choanae; around 52 parasphenoid teeth arranged in two series as inverted “V” shape; nasolabial protuberances are poorly developed (Fig. 3). Extremely well-marked costal grooves 13, counting one each in axilla and groin (Fig. 2). Tail is relatively short (TL/SL= 0.69) and robust with basal constriction and tip rounded. Limbs are robust and relatively long (combined limb length/SL= 0.5). Digits are well defined, relatively short, stout and triangular. Digits in order of decreasing length III-II-IV-I on hands and III-IV-II-V-I on feet (Fig. 2D). Phalangeal formulae 1-2-3-2 for hands and 1-2-3-3-2 for feet.

Coloration in life. Dorsum uniformly dark, color Vandyke Brown (282) from tail to head (including hands and feet). Prominent eyelids, jaw margins and gular surface pale Cinnamon-Rufous (31). Iris Raw Umber (280). Distal half of flanks dark, like dorsal coloration; proximal half Deep Vinaceous (248) extending to the venter and ventral side of limbs (Fig. 2A–2F).

Coloration in alcohol. Coloration very similar to that in life. Dorsum, dorsal surface of head, trunk, limbs, and tail Dusty Brown (285). Eyelids Raw Umber (280), slightly darker than in live individuals. Jaw margins and gular region Dark Grayish Olive (245). Distal half of flanks same color as dorsum, proximal half Deep Vinaceous (248), extending onto ventral surface. Ventral side of the tail color slightly more grayish than rest of its dorsum. Underside of hands and feet paler than in life (Fig. 2G–2H).

Measurements of holotype/paratype (in mm). Standard length (SL: distance from snout to posterior end of vent) 71.9/85.3; head width (HW) 15.8/17.7; head length (HL: snout to gular fold) 16.3/20.8; head depth at angle of jaw 6.8/7.9; eyelid length 5.3/6.2; eyelid width 2.9/3.5; anterior rim of orbit to snout 3.6/4.5; eye diameter 2.8/3.3; interorbital distance 4.5/5.0; snout to forelimb 21.2/27.4; internarial distance 4.5/5.5; intercanthal distance 4.8/5.6; nostril diameter 0.4/0.6; snout projection beyond mandible 1.5/1.5; snout to anterior angle of vent 65.9/82.1; axilla to groin 31.6/35.1; tail length (TL) 48.3/60.2; tail width at base 7.0/7.5; tail depth at base 8.9/9.8; forelimb length 16.2/20.0; hind limb length 20.0/25.7; hand width 5.9/6.5; foot width 7.5/8.7; length of the longest (third) toe 2.1/2.3; length of fifth toe 0.9/1.2; mental gland width 6.1/6.2; mental gland length 3.8/3.8; Numbers of teeth: premaxillary 6/10; maxillary 62/60; vomerine 46/42 (Fig. 3).

Distribution and Ecology. *Isthmura corrugata* is known only from the type locality, near the city of Coatepec in the mountainous region of central Veracruz (Fig. 4). This mountain range lies within the eastern portion of the Trans-Mexican Volcanic Belt. The vegetation at the type locality is cloud forest, characterized by the presence of tall trees that provide a high percentage of canopy cover, a deep layer of leaf litter (~8cm), and a high density of bromeliads and epiphytes. The dominant tree species are *Clethra mexicana*, *Hedyosmum mexicanum*, *Miconia* spp, *Platanus mexicana*, *Quercus* spp, as well as tree fern species (García-Franco *et al.* 2008; Toledo-Garibaldi & Williams-Linera 2014). A frequent fog maintains high levels of humidity. The holotype was found active on the forest floor at night, whereas the paratype was encountered inactive under a layer of leaf litter (~10 cm) during the day. Despite of *I. corrugata* has been found only in one locality, it is possible that this species may inhabit additional localities around Cofre de Perote extending to the Sierra Norte de Puebla near Cuetzalan, because a high proportion of the amphibian fauna is shared between these areas.

Six species of salamanders have been found in sympatry with *Isthmura corrugata*: *Aquiloerycea cafetalera*, *Parvimolge townsendi*, *Thorius pennatulus*, *Pseudoeurycea lynchi*, *Chiropterotriton chiropterus* and *Chiropterotriton* sp. Most of these species are found in the leaf litter, except for the last three, which also use arboreal bromeliads.

Etymology. The specific epithet makes reference to the extremely pronounced grooves along the body, giving these salamanders a corrugated appearance.

Discussion

The new species increases the number of species in the Mexican endemic genus *Isthmura* to seven. *Isthmura corrugata* is most closely related to *I. boneti* and *I. maxima*, both distributed in southern Mexico, specifically in the highlands around Oaxaca City and in portions of the Sierra Madre del Sur of Oaxaca and Guerrero, respectively. However, the new species is geographically much closer to *I. gigantea* and *I. naucampatepetl*, which are found in the southern portion of the Sierra Madre Oriental. In fact, the three species occur on the slopes of Cofre de Perote, Veracruz. No other region has three species of *Isthmura* in such close proximity.

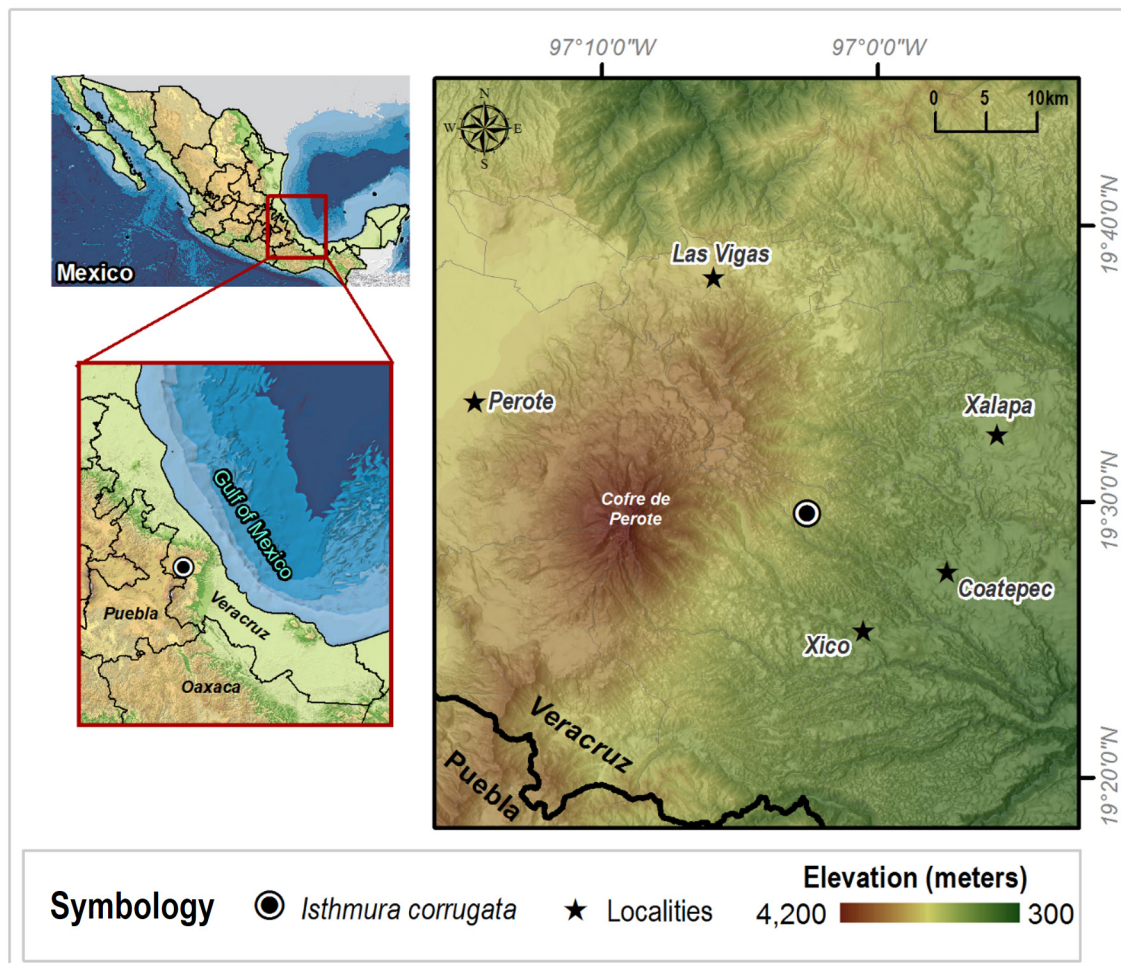


FIGURE 4. Map of the type locality of *Isthmura corrugata* in the mountainous region of central Veracruz, Mexico and, habitat at the type locality. Photograph by R. Luría (left) and A. Sandoval-Comte (right).

Cases of species that are geographically close, with one of them being phylogenetically closer to another geographically distant species, have already been documented in other plethodontid salamanders. In the genus *Thorius*, *T. pennatulus* occurs in central Veracruz and is the sister species of *T. smithi*, which occurs in Sierra de Juárez, Oaxaca, and is nearly sympatric with *T. adelos* and *T. insperatus*, both phylogenetically more distant (Rovito *et al.* 2013). Further studies are necessary to examine possible causes of this interesting pattern in some plethodontid species and the mechanisms involved.

The genus *Isthmura* contains species formerly included in the *Pseudoeurycea bellii* species group (Parra-Olea *et al.* 2005). It was named as a subgenus of *Pseudoeurycea* by Dubois & Rafaëlli (2012) and contained both the species of the *P. bellii* species group as well as the *P. cephalica* group (now the genus *Aquiloerycea*) until elevated

to genus level by Rovito *et al.* (2015). Species of *Isthmura* share at least two evident characters: 1) they are exclusively terrestrial salamanders with stout bodies and large size (SL to 161 mm), including the largest bolitoglossine species (*I. gigantea*) and 2) they have dorsal coloration that consists of a black background with a series of red to pinkish spots or chevrons (Parra-Olea *et al.* 2005). In this context *Isthmura corrugata*, in spite of sharing the character of large size (SL= 85 mm) with its congeners, is distinct in its color pattern. Its feet, with triangular toes, also differ from the typical foot morphology of other species of *Isthmura*.

The current information about natural history or population size of *I. corrugata* is not enough to assess its conservation status. However, the type locality has been sampled approximately 20 times by our team between 2010 and 2015, with a cumulative sampling effort of *ca.* 780 person-hours (Sandoval-Comte *et al.* 2012; Tobar-Suárez 2012; Hernández-López 2014; Meza-Parral & Pineda 2015; Oropeza-Sánchez 2016) and just two individuals have been recorded. This suggests that *I. corrugata* is rare at the site or that its behavior and habits make it difficult to detect. Although the type locality is located within a municipal protected reserve of about 105 ha, this area is threatened by logging and agricultural activities in neighboring areas and even in some portions within the reserve.

The only known locality of *I. corrugata* is within a broader area of about 800 km², on the eastern slope of Cofre de Perote (delimited by historical localities of amphibians collection around Xalapa, Naolinco, Teocelo, and Las Vigas towns, between coordinates 19°40'03" and 19°21'49" N, and 97°7'43" and 96°50'48" W), where 19 plethodontid species from seven genera have been recorded (see Wake & Lynch 1976; Wake *et al.* 1992; Parra-Olea *et al.* 2001; Parra-Olea *et al.* 2010). Twelve of these 19 salamander species are in high-risk categories, according the IUCN's Red List (2016), and the region is currently dominated by transformed environments such as agricultural crops, cattle pastures, human settlements, among other types of land use, and only 23% is covered by cloud forest and temperate forest (Castillo-Campos *et al.* 2011). This region is of high priority for the conservation and the study of plethodontid salamanders.

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