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New records and distribution extension of the rare glassfrog *Hyalinobatrachium chirripoi* (Anura: Centrolenidae) throughout the Chocó-Magdalena region in Colombia

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Hyalinobatrachium is the most diverse glassfrog genus (family Centrolenidae) with 32 species described to date, ranging from Mexico to Argentina (Guayasamin et al. 2009; Frost 2019). Given the low levels of morphological differentiation within this genus, species identification is sometimes difficult, and requires the use of alternative sources of evidence such as molecular phylogenetics and DNA barcoding (Castroviejo-Fisher et al. 2009). *Hyalinobatrachium chirripoi* (Taylor 1958) is a seldom observed species found in forests under 600 m elevation from Honduras, along the Chocó-Darien to the Esmeraldas Province in north Ecuador (Kubicki 2007; Guayasamin et al. 2016). Here new records of *H. chirripoi* are reported which extend the distribution of this species into the Andean foothills of the central Chocó and, for the first time, into the Magdalena Valley of Colombia. An overview of the known distribution *H. chirripoi* is presented, including previous museum records and the new data.

Specimens examined. One individual was collected in 2010 at Vereda El Porton, in San Francisco, Antioquia, Colombia (5.9015, -74.96925, 589 m asl; Fig. 1), in the Magdalena River drainage. The individual was found at night, calling on the underside of a *Heliconia* leaf overhanging a small stream in a secondary forest, and was euthanized with an overdose of 2% Roxicaine and fixed in 10% formalin. A liver sample was preserved in 99% ethanol. The specimen was deposited in the Museo de Herpetología, Universidad de Antioquia, Colombia (voucher MHUA-A 06650; originally misidentified as *H. fleischmanni*). In 2016, two other individuals were collected during nocturnal surveys performed at 20 km SW of Condoto, Chocó, Colombia, on the western versant of the Cordillera Occidental (5.02078, -76.51633, 423 m asl; Fig. 1A). They were found calling from the upper side of leaves in a tree hanging above a small river (Fig. 1B). They were captured and euthanized with an overdose of topical lidocaine hydrochloride (Xylocaine). Muscle samples were stored in 100% ethanol. Specimens were then fixed with 100% ethanol and deposited in the herpetology collection of the Natural History Museum at Universidad de los Andes, Colombia (vouchers ANDES-A3738 and ANDES-A3739). Other individuals at the same locality were observed on leaves and fronds of Araceae, Musaceae (*Heliconia*), and ferns (Polypodiaceae), perched ~3–8 m off the ground. Both localities (Vereda El Porton at the Magdalena River drainage, and 20 km SW of Condoto in the Chocó) are classified as tropical wet forest biome (bh-T, Holdridge 1964).

Morphological and molecular identification. These samples were identified as *H. chirripoi* based on light dorsal spots, significant webbing between Fingers II and III, clear parietal peritoneum, bare heart condition (i.e., iridophores covering all visceral peritonea except for the urinary bladder and pericardium), tympanum visible, and a truncate snout in sagittal view (Taylor 1958; Ruiz-Carranza and Lynch 1998; Savage 2002, Fig. 2). To

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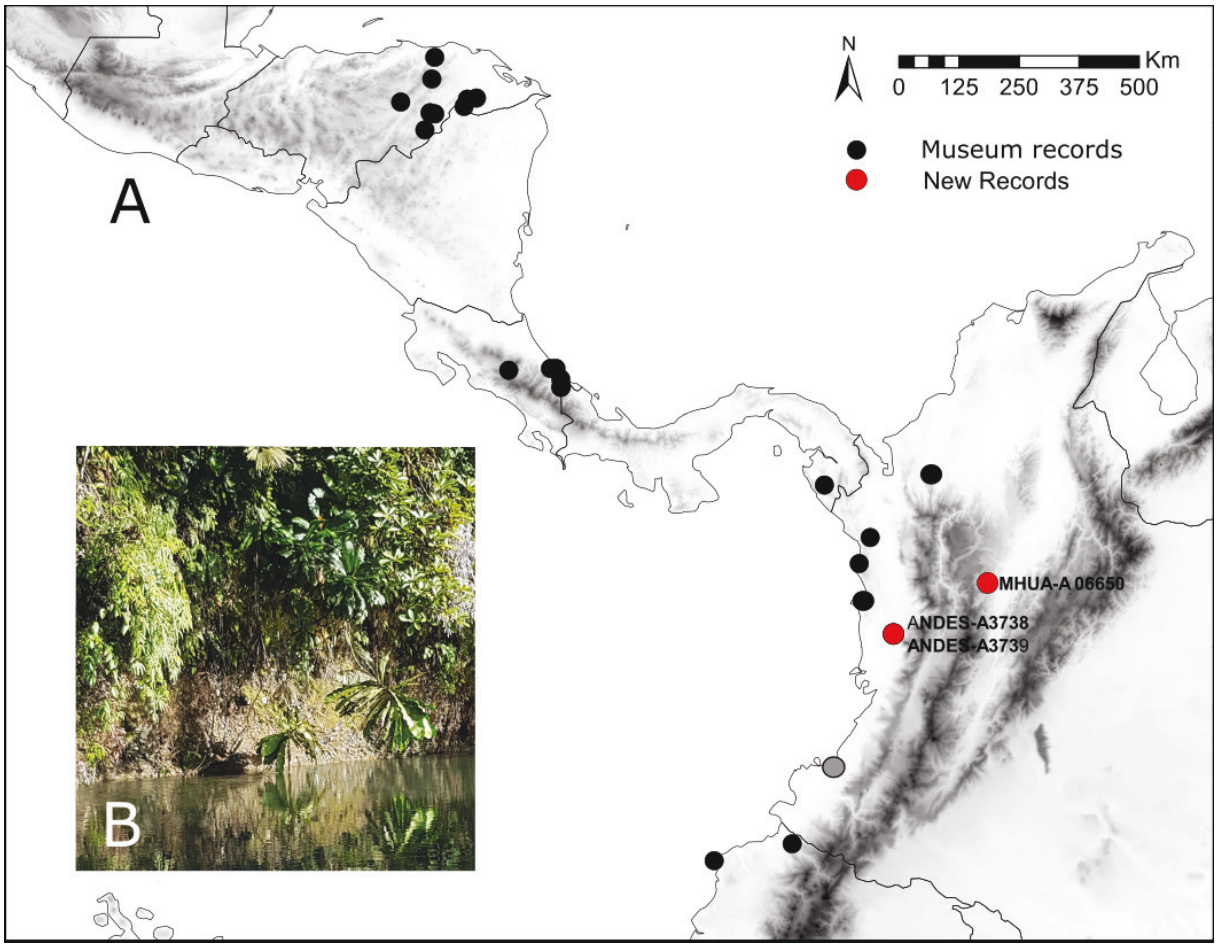

Fig. 1. **(A)** Localities of museum specimens (black dots) and new records (red dots) for *Hyalinobatrachium chirripoi.* Coordinates for specimen IAvH-A-4311 (gray dot) were approximated to the urban area of Río Guapi, Cauca, since precise coordinates of the collection site were lacking. **(B)** Habitat where the ANDES-A individuals were encountered.

further corroborate morphological diagnoses, mtDNA barcoding was used. DNA was extracted following Ivanova et al. (2006) for specimens ANDES-A3738 and ANDES-A3739, or using the Thermo Scientific DNA extraction kit for specimen MHUA-A 06650. Amplification of 16S (567 bp) and COI (609 bp) loci was as described by Guayasamin et al. (2008), and Mendoza et al. (2016; primers from Meyer et al. 2005), respectively. Purified products were Sanger-sequenced in both directions. Sequences obtained are deposited in GenBank under accession numbers MH129045–49.

Sequences of both genes were blasted against the GenBank non-redundant database using megaBLAST. COI sequences were also used as input for the BOLD DNA barcoding system (Ratnasingham and Hebert 2007). In addition, Kimura-two-parameter (K2P; Kimura 1980) pairwise distances between sequences of closely related *Hyalinobatrachium* species available in GenBank (Table 1) were calculated using MEGA7 (Kumar et al. 2016) and maximum likelihood and Bayesian mtDNA genealogies were built with RAxML v.8.2.10 (Stamatakis 2006, 2014), and MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003, Ronquist et al. 2012), respectively.

Maximum likelihood searches used the rapid hill-climbing algorithm and 10,000 rapid bootstrap pseudo-replicates to assess nodal support. In MrBayes two independent 2,000,000 generation analyses were run, sampling every 1,000 generations, and with 20% burn-in. The best models for molecular evolution for the 16S and for each codon position of the COI gene were selected using PartitionFinder 2 (Lanfear et al. 2016).

Mitochondrial sequences unambiguously confirmed the identity of the specimens as *H. chirripoi*. All BLAST searches against GenBank returned *H. chirripoi* sequences as the top hit, with 99% identity and e-values of zero. Online BOLD identification searches matched the COI sequences to *H. chirripoi* with 99.3–99.5% identity. On the other hand, *H. fleischmanni* sequences matched the query sequences with 83.8% similarity (BOLD) and 83.6% identity (GenBank). Maximum likelihood and Bayesian trees corroborated these results, with the query sequences nested within a well-supported clade that includes all the other *H. chirripoi* (Fig. 3). Finally, K2P distances among *H. chirripoi* samples averaged 0.006 (range = 0.002–0.009) for 16S and 0.027 (0–0.039) for COI, while the mean distance with *H. colymbiphyllum*, its sister species, was 0.021 (0.018–0.024) for 16S and 0.081 (0.059–0.093) for COI.

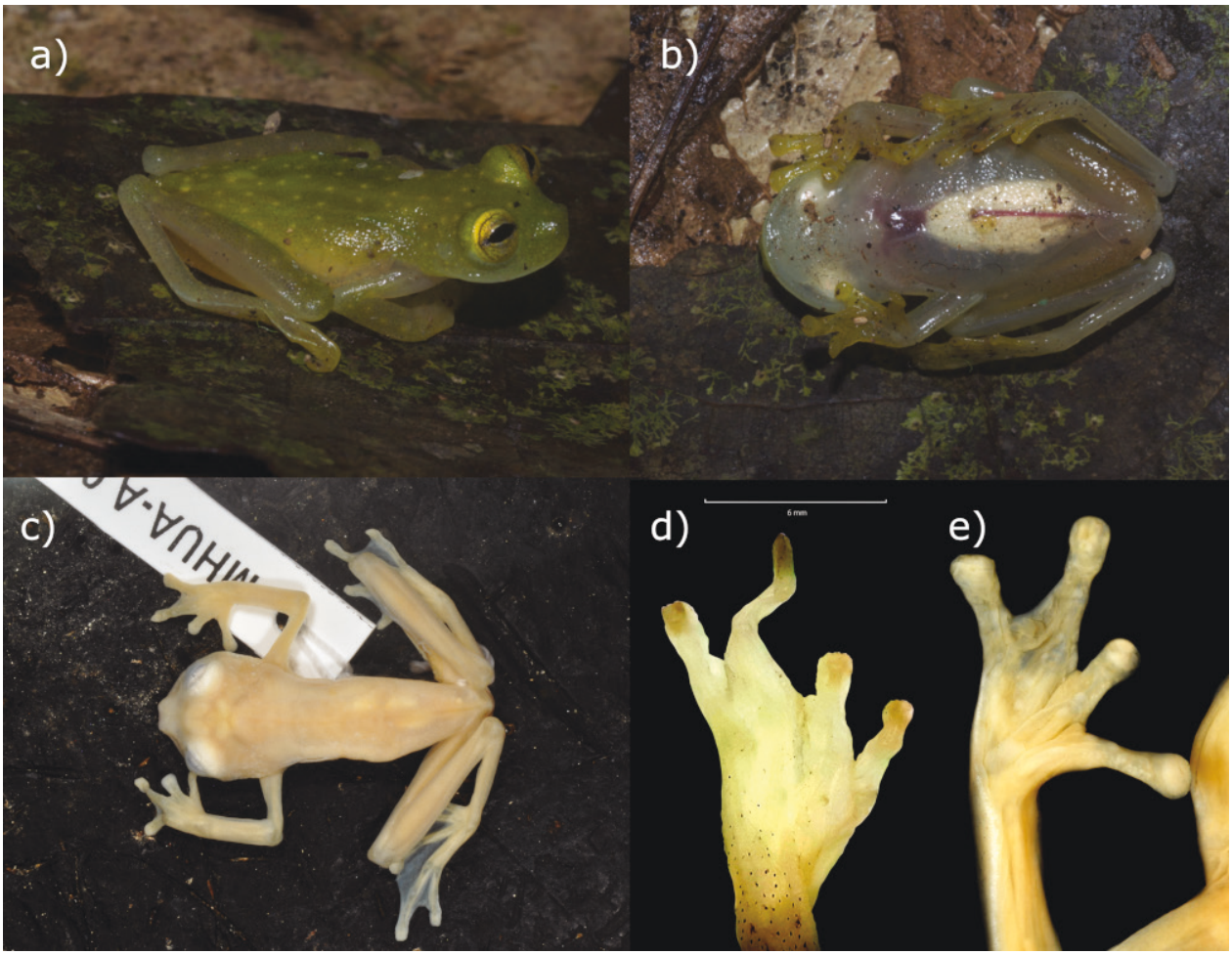

Fig. 2. Dorsal **(a)** and ventral **(b)** view of live specimen from Chocó-Darien (ANDES-A-3738). Dorsal view **(c)** of specimen collected in Madgalena basin (MHUA-A-6650). Details of hand webbing of specimens collected in Chocó-Darien **(d)** and Madgalena basin **(e)**.

Distribution and conservation implications. The records of *H. chirripoi* since its rediscovery by Kubicki (2004) are very scarce. In Colombia there have been very few isolated records of the species (Hayes and Starret 1980; Romero-Martínez et al. 2008; Ruiz-Carranza and Lynch 1998). Most previous records for the species in Colombia are restricted to the Northwest Chocó-Darien region close to Panama, in Nuquí (MHUA-A 5150-53), and in Bahía Solano (ICN 40270-314) (Fig. 1). One additional specimen was collected in 1987 further south, near Río Guapi, Cauca (specimen IAvH-Am-4311) with no georeferenced locality (gray circle in Fig. 1) and the southernmost specimens were collected from Esmeraldas Provinces in Ecuador (QCAZ-A 48271, QCAZ-A 66603, Guayasamin et al. 2016). The new records reported here fill the gap in the Chocó-Darien between the Bahia Solano and Rio Guapi records, extending the distribution of this species 70 km into the Chocoan mainland and into the foothills of the Western Andes (400 m asl).

These records also extend the distribution of *H. chirripoi* into the Magdalena basin, across the Andes from all previous records of this species. Previously, the closest record of *H. chirripoi* to the Magdalena basin was from the Cerro Murrucucú in Tierralta, Córdoba, within the Parque Nacional Natural Paramillo (ICN 39129–30), an intermediate zone between Chocó-Darien rainforests and Magdalena basin. This region is included in the Sinú-San Jorge District, characterized by a biota with common elements, including several amphibian species of the Chocoan, Amazonian, and Magdalenian regions (Henao-Sarmiento et al. 2008; Hernández-Camacho et al. 1992a; Marquez et al. 2017; Romero-Martínez et al. 2008; Vasquez and Serrano 2009), and is considered as a transition zone between the Chocó-Darien, Caribbean, and Magdalena bioregions (Romero-Martínez et al. 2008). Congruently, the Magdalena basin record reported here lies within the Nechí District, for which the biological elements have affinity with those from the upper Sinú and high San Jorge drainages, as well as the Chocó-Darien region (Hernández-Camacho et al. 1992b).

With these new records of *H. chirripoi*, the Magdalena basin and Chocó-Darién regions in Colombia share a total of five species of the genus (including *H. fleischmanni*, *H. colymbiphyllum, H. aureoguttatum*, and *H. valerioi*). *Hyalinobatrachium aureoguttatum* and *H. valerioi* are easily differentiable by the dorsal coloration (large yellow round spots on a green background), but

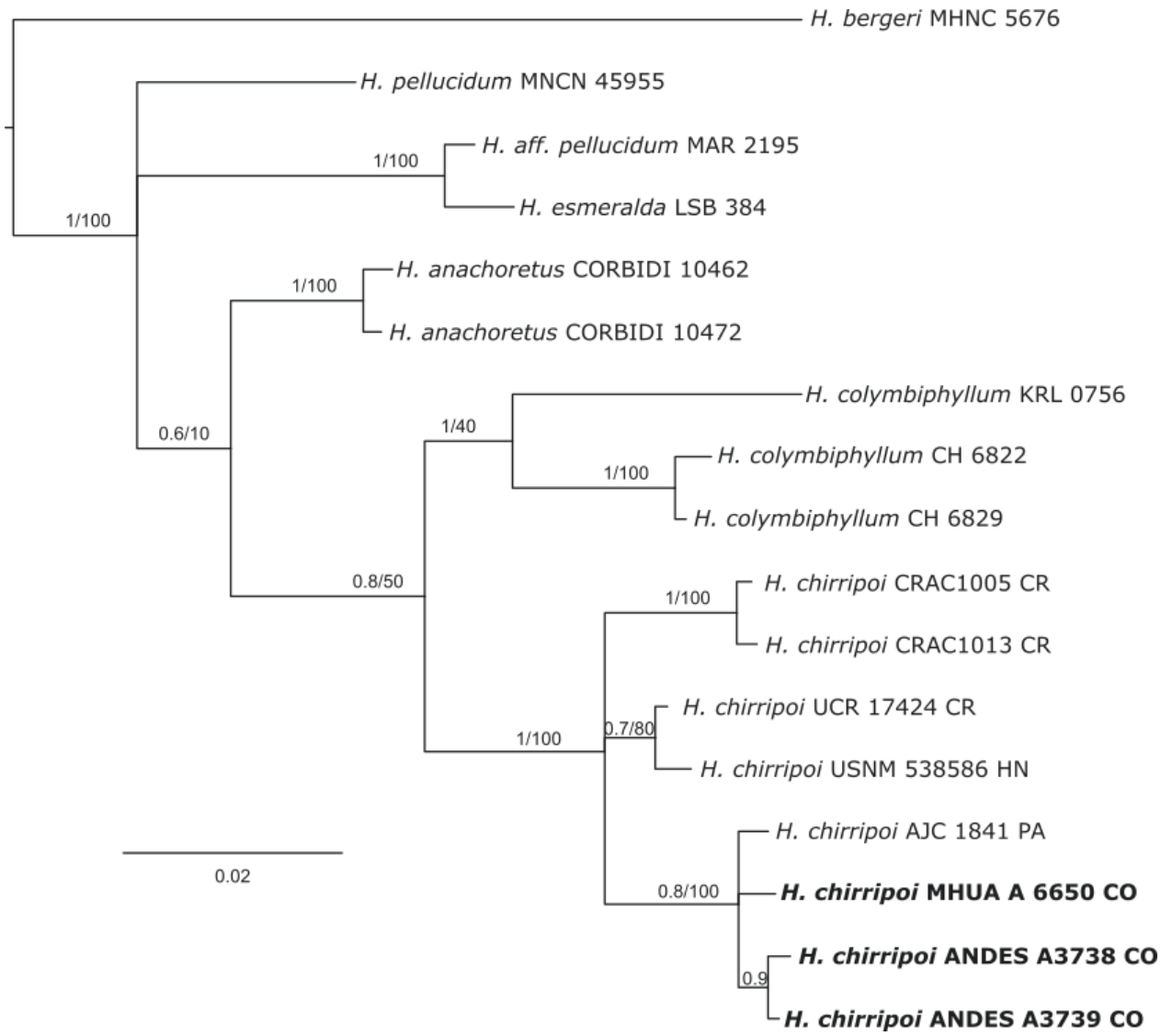

Fig. 3. Phylogenetic positions of three *Hyalinobatrachium chirripoi* samples from Chocó and Antioquia, Colombia, in a Bayesian mtDNA tree inferred from 16S rRNA and COI sequences. The chosen models of evolution using Partition finder were: 16S: GTR+I, COI position 1: GTR+I, position 2: SYM+G, and position 3: F81+I. Samples in bold are from this study, while the others are from GenBank. Posterior probability and bootstrap support values (from a maximum likelihood analysis) are indicated in front of the corresponding node as PP/Bootstrap. Two letter country codes provided for *H. chirripoi* samples follow the International Organization for Standardization: CO = Colombia, PA = Panama, HN=Honduras, CR = Costa Rica.

misidentification is common for the other three species (Kubicki 2004). The most relevant external feature for differentiating *H. chirripoi* is the extensive webbing between Fingers II–III (*H. colymbiphyllum* and *H. fleischmanni* have little webbing between Fingers II–III); additionally *H. fleischmanni* has iridophores covering the pericardium, while *H. chirripoi* and *H. colymbiphyllum* lack iridiophores in the pericardial peritonea (Savage 2002; Starret and Savage 1973, but check Cisneros-Heredia and McDiarmid 2007). After a detailed revision of the *H. fleischmanni* specimens for the Magdalena basin stored in the Museo de Herpetología of Universidad de Antioquia, no additional misidentified *H. chirripoi* were found. However, this work highlights the importance of carefully inspecting museum specimens of *Hyalinobatrachium* (and other taxa with low morphological differentiation between species) when using such specimens for biogeographic and conservation work, in order to avoid errors associated with misidentification.

A shortage of information still remains on the amphibian diversity in Chocó-Darien rainforest and Magdalena basin, both of which are increasingly threatened by human activities such as mining, habitat loss, fragmentation, and other forms of landscape transformation (Etter and van Wyngaarden 2000; Rangel 2004). Indeed, according to the IUCN Red List, certain populations of *H. chirripoi* in Panama and Colombia are threatened by habitat loss, due to increasing agricultural activity and logging (Solís et al. 2008). The new records presented here provide additional information about the distribution of this rare species, and highlight the

Table 1. Sequences for mitochondrial regions 16S and COI of *Hyalinobatrachium chirripoi* and related species used in this study.

Species	Voucher	16S	COI
H. chirripoi	ANDES-A3738	MH129045	MH129047
H. chirripoi	ANDES-A3739	MH129046	MH129048
H. chirripoi	MHUA-A-6640	MH129049	NA
H. chirripoi	UCR 17424	EU663037	NA
H. chirripoi	USNM 538586	EU663038	NA
H. chirripoi	AJC 1841	KF604299	KF604294
H. chirripoi	CRAC1005	NA	KJ703104
H. chirripoi	CRAC1013	NA	KJ703105
H. bergeri	MHNC 5676	EU663033	NA
H. pellucidum	MNCN 45955	KM068262	NA
H. esmeralda	LSB 384	KP149361	KP149161
H. colymbiphyllum	KRL 0756	FJ784359	NA
H. colymbiphyllum	CH 6829	KR863254	KR862999
H. colymbiphyllum	CH 6822	KR863256	KR863001
H. anachoretus	CORBIDI 10462	KM068268	NA
H. anachoretus	CORBIDI 10472	KM068300	NA
H. aff. *pellucidum*	MAR-2195	KM068296	NA

importance of using an integrative taxonomic approach at the junction between these two bioregions in terms of biodiversity conservation, as well as the need for continued documentation of their biological richness.

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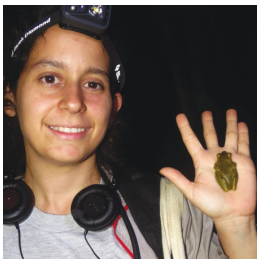

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