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Research Article

Phylogeny-based species delimitation and integrative taxonomic revision of the *Hyalinobatrachium fleischmanni* species complex, with resurrection of *H. viridissimum* (Taylor, 1942)

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Hyalinobatrachium fleischmanni is one of the widest ranging glassfrog species, occurring in the lowlands from Mexico through Central America to Ecuador. Despite its conservative morphology, previous studies suggested that the species is comprised of multiple lineages. Here we test the hypothesis of cryptic species within *H. fleischmanni* by means of morphology, morphometrics, bioacoustics, and molecular analysis. Molecular delimitation based on mitochondrial and nuclear genes detected 17 candidate species within *H. fleischmanni* but combined with other sources of evidence, we support the recognition of at least three different species within the name *H. fleischmanni*. The identity of *H. fleischmanni* sensu stricto is supported for populations from Costa Rica to eastern Honduras while the name *Hyalinobatrachium tatayoi* corresponds to the southern lineages in Costa Rica and South America. Those two species differ in the note duration of the advertisement call and in the absence of nuptial pads in the hand webbing of *H. fleischmanni* males. Populations from Mexico and Guatemala represent a third species to which we assign the available name *H. viridissimum* **comb. nov.** *Hyalinobatrachium viridissimum* differs from *H. fleischmanni* and *H. tatayoi* in mitochondrial DNA divergence, variation in peak frequency, and note duration of the advertisement call. A divergent lineage from western and central Honduras is tentatively assigned to *H. viridissimum*. Based on these results, we provide updated information for each species.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:96D65174-4F8B-4E4B-8C85-967767311255

Key words: Bioacoustics, Centrolenidae, glassfrogs, integrative taxonomy, species delimitation

Introduction

Hyalinobatrachium is a taxonomically stable genus of glassfrogs (family Centrolenidae) with 33 currently recognized species (Frost, 2019; Guayasamin et al., 2009), distributed in tropical Mexico and Central America, the tropical Andes, the Coastal Cordillera of Venezuela, Tobago, the Amazon Basin, and the Guiana Shield (Guayasamin et al., 2009). Due to the morphological similarities among the species within the genus (Señaris & Ayarzagüena, 2005), species identification and delimitation has been challenging, and taxonomic studies have required a molecular approach (Castroviejo-Fisher et al., 2011; Mendoza et al., 2019b).

The type species of the genus, *Hyalinobatrachium fleischmanni*, is one of the most studied centrolenid frogs (e.g., Delia et al., 2010; Starrett & Savage, 1973; Villa, 1984) due to its putatively widespread distribution from Mexico to northern South America. The limited morphological variation for certain characters

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(colouration, morphometry) combined with the high variation of other characters (guanophores of the pericardium, interdigital webbing; Cisneros-Heredia & McDiarmid, 2007) led to the recognition of a single species with a very large geographic distribution. Through time, multiple names have been synonymized with *H. fleischmanni*, including *Hylella chrysops* (Cope, 1894), *Cochranella decorata* (Taylor, 1958) and *Cochranella millepunctata* (Taylor, 1958) from Costa Rica, and *Centrolenella viridissima* (Taylor, 1942) from Mexico. On the other hand, some populations previously considered to represent *H. fleischmanni* have been recognized as distinct species, such as *H. guairarepanense* in Venezuela (Barrio-Amorós, 2004). Duellman and Tulecke (1960) stated that all Mexican material represented a single species, and that the characteristics used by Taylor (1942) for the description of *C. viridissima* were variable and overlapped to a considerable extent with Costa Rican *H. fleischmanni* but nonetheless recognized *C. viridissima* as tentatively distinct. Later, Starrett and Savage (1973) synonymized *C. viridissima* with *H. fleischmanni*, arguing that populations considered to represent both taxa are connected by a continuous series of populations through Central America.

Castroviejo-Fisher et al. (2007) described *H. tatayoi* from a single locality in the Serranía del Perijá in Venezuela, based on subtle differences in morphology and acoustics with respect to Central American populations of *H. fleischmanni*. Subsequent molecular studies (Castroviejo-Fisher et al., 2009; Delia et al., 2017; Guayasamin et al., 2008) suggested that *H. fleischmanni* was paraphyletic with respect to *H. tatayoi.* Recent phylogeographic analysis of *H. fleischmanni* based on mitochondrial DNA across its entire distribution identified three large genetically divergent clusters (Mendoza et al., 2019a). Samples from the type locality of *H. fleischmanni* are clustered in a Central American small-range clade (the Central clade of Mendoza et al., 2019a) that includes populations from southern Costa Rica, Nicaragua, and eastern Honduras. The populations from Mexico, Guatemala, and west Honduras formed a second clade (the Northern clade), formed by four allopatric lineages, referred to as the Maya, Gulf, Pacific, and West Chortis clades by Mendoza et al. (2019a). Finally, populations from Panama and South America, including those assigned to *H. tatayoi*, formed a third clade, the Southern clade.

Herein, we re-evaluate the taxonomy of *H. fleischmanni* and *H. tatayoi* by combining three lines of evidence: genetic (nuclear and mitochondrial), acoustics, and morphology to detect lineage divergence and to determine the identities and geographic distribution of each valid species. Based on our results, we assign the name *H. tatayoi* to the Southern clade from Costa Rica and South America, and we resurrect the name *C. viridissima* (as *H. viridissimum* **comb. nov.**) and assign it to all four lineages of the Northern clade, with the caveat that further investigation is needed to determine the distinctiveness and taxonomic status of each of the four lineages within the Northern clade.

Materials and methods

Species criterion

Our definition of species follows the general metapopulation lineage species concept (deQueiroz, 2007; Simpson, 1951). We recognize a species when there is evidence for the separation of metapopulation lineages, preferably based on multiple lines of evidence, following the consensus protocol for integrative taxonomy (Dayrat, 2005; Padial et al., 2010). We used two criteria to delimit species boundaries using molecular data: reciprocal monophyly and genetic distances (reviewed in Vences & Wake, 2007). The first criterion is based on the assumption that coalescent patterns in gene genealogies are related to historical processes that originate separate lineages (e.g., Avise, 2000; Knowles & Carstens, 2007). The second assumes that genetic divergence between populations within a species tends to be relatively small because of gene flow, whereas divergence between species increases with time.

Phylogenetic analysis

We used a subset of 56 previously published samples from the entire distribution of *H. fleischmanni* and *H. tatayoi* and four outgroups for phylogenetic reconstructions (Mendoza et al., 2019a). This subset included sequences for three mitochondrial genes (16S, COI, and ND1). Since an analysis based only on the mitochondrial genome may display differences between gene and species trees as a result of distinct biological processes, such as mitochondrial DNA introgression, a nuclear gene (POMC) was included for 38 of those samples, following laboratory protocols by Guayasamin et al. (2008). Mitochondrial and nuclear sequences were concatenated to perform new Maximum likelihood (RAxML) and Bayesian reconstructions (Beast and MrBayes) following the methods outlined in Mendoza et al. (2019a). The new sequences were deposited in GenBank (accession numbers: MK817126–MK817164).

Analysis of molecular species delimitation

From the phylogenetic trees obtained with the entire set of sequences (nuDNA + mtDNA), we used three approaches to test species boundaries. First, we employed the general mixed Yule-coalescent (GMYC, Pons et al., 2006) model to estimate species boundaries using the ultrametric tree. The GMYC method (Fujisawa & Barraclough, 2013) uses a speciation and a neutral coalescent model. It strives to maximize the likelihood score by separating/classifying the branches of an ultrametric tree (in units of absolute or relative ages) into two processes; within and between species, searching the transition point between species-level and population-level variability based on a shift in the rate of an ultrametric tree branching (Pons et al., 2006). These analyses were performed with single and multiple thresholds for the ultrametric tree in the splits package (Ezard et al., 2009) in R version 3.5.1.

Second, we used the Bayesian Poisson tree process (bPTP; Zhang et al., 2013) and the multi-rate PTP (mPTP) model (Kapli et al., 2017) in the web servers (https://species.h-its.org/ptp/ and https://mptp.h-its.org respectively) on multilocus phylogenetic trees inferred by MrBayes and BEAST. The bPTP was run for 100,000 generations, with a thinning of 100 and a 10% burn-in. These two analyses use the number of substitutions to identify significant changes in branching rates in a phylogeny, thereby they do not depend on the accuracy of ultrametric tree estimations. In addition, it may outperform other species delimitation methods when evolutionary distances are small (Malavasi et al. 2016; Pons et al., 2006). In particular, mPTP incorporates different coalescence rates between clades, allowing for different levels of intraspecific genetic diversity (Kapli et al., 2017).

Third, we used a coalescent-based modelling approach called Bayesian Phylogenetics and Phylogeography (BP&P v.2.0; Yang & Rannala, 2010) to generate the posterior probabilities of species assignments taking into account uncertainties due to unknown gene trees and the ancestral coalescent process. BPP evaluates speciation models using a reverse jump Markov chain Monte Carlo (rjMCMC) algorithm to determine whether to collapse or retain branches throughout the phylogeny.

Following Leaché and Fujita (2010) we applied the following assumptions on population sizes and divergences: (1) Large ancestral population sizes and deep divergences (theta ~ G(1, 10) and t0 ~ G(1, 10), both with a prior mean = 0.1 and variance = 0.01; (2) Relatively small ancestral population sizes and shallow divergences among species theta ~ G(2, 2000) and t0 ~ G(2, 2000), with a prior mean = 0.001 and variance = 5×1027); and (3) Large ancestral populations sizes (theta ~ G(1, 10) and relatively shallow divergences among species t0 ~ G(2, 2000). Analyses were run for 500,000 generations (first 10,000 were burn-in), with a sampling interval of 5. We used the BEAST topology as a guide species tree, and the number of candidate species was based on the PTP results as input. A conservative approach was used for the BP&P and bPTP analysis; we required strong support (pp $\geq$ 0.95) across all runs to retain a given branch (i.e., indicating lineage splitting).

Finally, we compared the genetic distances from the lineages obtained in the delimitation analysis (based on strict consensus) with those from recent studies in glassfrogs (i.e., Amador et al., 2018; Rada et al., 2017) and DNA barcoding in anurans (Lyra, Haddad, & de Azeredo-Espin, 2017). To do so, we calculated the uncorrected p-distances for 16S and COI genes in MEGA version 7 (Kumar et al., 2016).

Morphology

Following Castroviejo-Fisher et al. (2011), we considered morphology to be divergent if a taxon exhibits at least one fixed (qualitative) or non-overlapping (quantitative) character or a unique combination separating it from each of the other taxa. The underlying assumption is that fixed differences in heritable morphological traits might be strong evidence of reduced or absent gene flow (Wiens & Servedio, 2000); thus, constituting evidence of independent lineages (deQueiroz, 2007).

We examined 174 specimens from the following institutions: Museo de Herpetología de la Universidad de Antioquia (MHUA-A, $n = 43$), American Museum of Natural History (AMNH, $n = 36$), Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ, $n = 19$), Universidad de Costa Rica (UCR, $n = 10$), Colección Nacional de Anfibios y Reptiles, Instituto de Biología, Universidad Nacional Autónoma de México (CNAR-IBH, $n = 24$), and Museo de Zoología de la Facultad de Ciencias (MZFC, $n = 42$). All specimens were measured by the same person (AMMH) except for those from Universidad de Costa Rica (EA). A list of material examined is provided in Appendix S1.

Each locality from museum databases was carefully checked (lat–long coordinates) to correct for imprecise geo-references in decimal degrees, based on the WGS 1984 datum. Ten standardized morphological measurements for centrolenids were selected following Cisneros-Heredia and McDiarmid (2007): Snout-vent length (SVL); Head length (HL); Head width (HW); Inter-orbital distance (IOD); Horizontal eye diameter (ED); Eye-nostril distance (ES); Width of disc on the

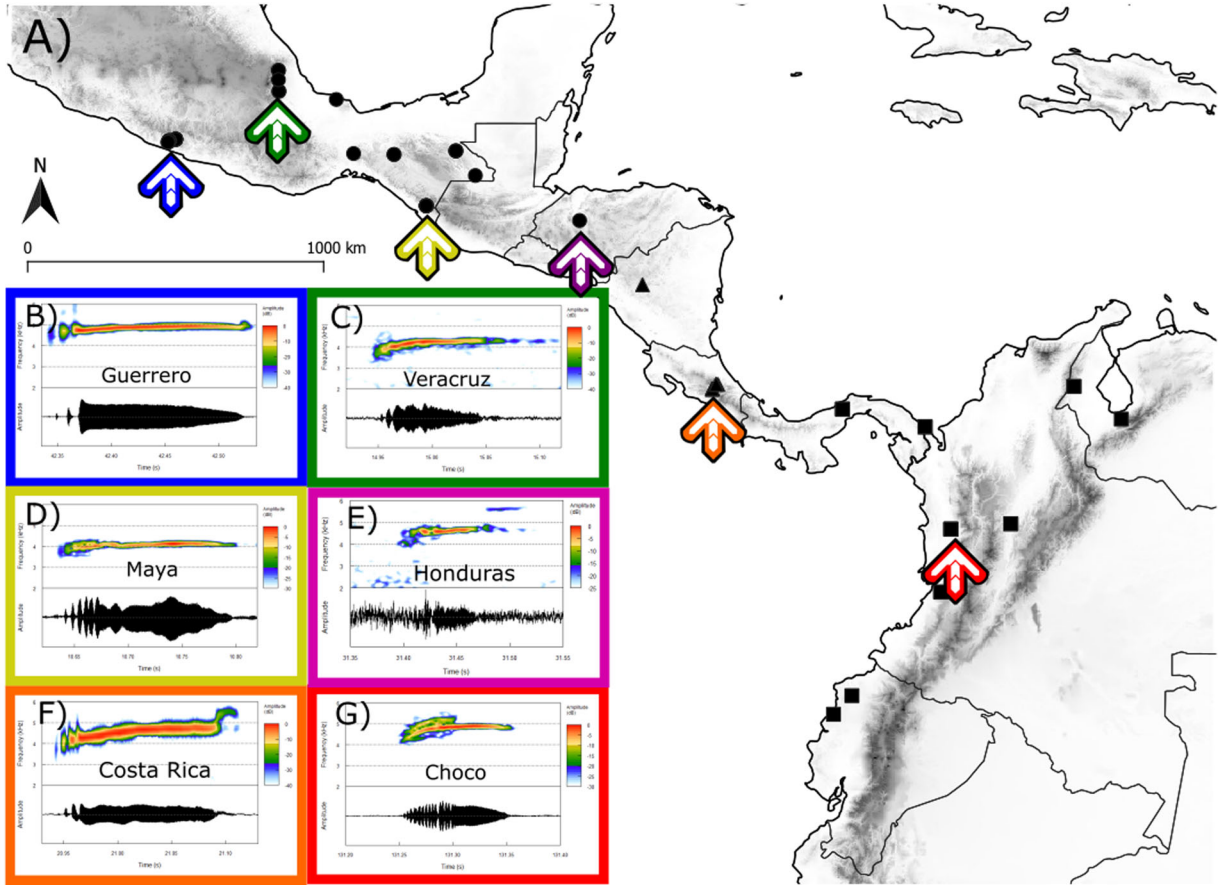

Fig. 1. (A) Location of the 64 calls for *Hyalinobatrachium fleischmanni* species complex analysed in this study. Each shape represents the candidate species tested (circle = *H. viridissimum* **comb. nov.**, triangle = *H. fleischmanni* and square = *H. tatayoi*), the colour of the arrow indicates the location of the corresponding spectrogram. (B–G) Spectrogram and sonogram of the advertisement call from some localities evaluated.

third finger (FIII); Femur length (FL); Tibia length (TL); Foot length (FL). The measurements were made with a digital calliper and rounded to the nearest 0.1 mm. Since our data do not show homogeneity of variances (based on a Levene's test), we compared morphometric variables between lineages with a Wilcoxon rank test. Subsequently, the dimensions of the scaled measurements were reduced by means of a Principal Component Analysis (PCA) on the residuals of the linear regressions between the SVL and the morphometric variables (to remove the effect of body size) and the groups generated from the first two components were visualized in a plot.

Additionally, 10 categorical characters suggested by Cisneros-Heredia and McDiarmid (2007) were also evaluated: Snout form at dorsal and lateral view, tympanum visibility, dorsal skin texture, cloacal ornamentation, colour of peritonea, shape of liver, hand webbing, iris colouration, and type of nuptial pads.

Bioacoustics

We employed differences in advertisement call since they are usually interpreted as evidence of lineage divergence that can be used to separate species (Bickford et al., 2007; Köhler et al., 2017; Padial et al., 2008; Vences & Wake, 2007). We considered that advertisement calls strongly indicate the existence of lineage divergence when they do not overlap in quantitative parameters, since vocalizations directly tied to mate recognition and sexual selection can be considered a prezygotic reproductive barrier, establishing and maintaining reproductive isolation (Vences & Wake, 2007).

Recordings of advertisement calls were obtained throughout the entire distribution of the species (Fig. 1) from collections and researchers (unpublished data) and were complemented by field recordings made in Colombia and Mexico through a Sennheiser unidirectional microphone (ME66/K6) connected to a Tascam DR-40 recorder. The recording from Honduras was

made using a Sennheiser MKE 400 microphone and Olympus WS-823 digital recorder.

At least 10 calls per individual were recorded in .wav format at a sampling frequency of 44.1 kHz and an amplitude resolution of 16 bits. The sound files were analysed in the software Raven Pro 1.4 (Cornell University, Ithaca, NY, USA). Voucher specimens were fixed with 10% formalin and deposited at the Colección Nacional de Anfibios y Reptiles (CNAR), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM) under the numbers IBH-31786 to 13809 (for the Mexican specimens) and at Colección de Anfibios from Instituto de Investigación de Recursos Biológicos Alexander von Humboldt under the numbers IAvH-Am-14756 to 14770 (for the Colombian specimens). The recordings were deposited in Colección de Sonidos Ambientales (CSA) of Humboldt Institute and the Colección digital de cantos grabados of Museo de Zoología de la Facultad de Ciencias, UNAM.

The spectrogram of each call was obtained from the Fourier transformation with a Blackman type window of 5 ms, with 80% superposition and a DFT of 1024 in the software Raven Pro 1.4 (Bioacoustics Research Program, 2013), and the following parameters taken: Peak frequency (frequency at maximum amplitude), length of the note (length in milliseconds of a note, where a note is a discrete series of pulses) and frequency bandwidth. We generated call figures using Seewave v. 1.6 package (Sueur et al., 2008) and in WarbleR (Araya-Salas & Smith-Vidaurre, 2017) in R (version 3.5.1) (R Core Team, 2019). The differences in the components of the song between the lineages were evaluated with a Kruskal–Wallis test and by a Wilcoxon rank-sum test. Subsequently, a 2D plot of spectral and temporal domain (peak frequency vs. call duration) was constructed as a visual inspection of acoustic space isolation.

Results

Phylogenetic analysis

The best-fit model for our dataset was HKY + I + G for 16S, GTR + G for COI, GTR + G + I for the first codon of POMC, TRNEF for the second codon of POMC, and HKY + X for the third codon for POMC. Our analysis with nuclear and mitochondrial genes clearly shows that *H. fleischmanni* is differentiated into genetic groups (Fig. 2). Two main, well-supported clades were recovered (pp $>$ 0.95 for most cases). The first clade was divided into two lineages: a large lineage containing all samples from Mexico and Guatemala (divided into three subgroups) and a smaller one containing samples from western Honduras and a small region of eastern Guatemala. The second clade is formed by two lineages, one includes samples from east Honduras to Costa Rica (including the type locality of *H. fleischmanni*) and the second with samples from Panama to Ecuador, including the type locality of *H. tatayoi* from Venezuela. Monophyly of these groups is supported by posterior probability (PP) $>$ 0.98 and is consistent with Bayesian and Maximum likelihood analyses of concatenated sequences.

Molecular delimitation

GMYCs and GYMCm approaches applied to the MrBayes reconstruction, suggested 17 candidate species from the *H. fleischmanni* sensu lato matrix (Fig. 2). The northern samples were grouped as six different candidate species. The likelihood value was higher in both approaches than the value for the null model (ML single = 164.22; ML multiple = 164.91; null L = 161.38), but the results of the likelihood ratio tests was significant only for the multiple analysis (LRTest = 0.058 ns for GYMCs and 0.029* for GYMCm) suggesting that multiple thresholds are necessary in different parts of the tree to delimit species.

The ML implementation of PTP and the Bayesian implementation of the method (bPTP) applied to the MrBayes tree delimited 8–11 putative species with high support values (0.99–1), for Beast and MrBayes reconstruction. The northern samples were recovered in at least four distinct lineages in all analysis and the results with Beast tree split the southern clade in three to five different species. Bayesian species delimitation on the concatenated data sets of all genes for all three scenarios support the guide tree with eight species with speciation probabilities of 1.0.

Thus, based on strict consensus of all molecular delimitation analyses here tested, our phylogenetic approach demonstrates that *H. fleischmanni* is genetically well structured into up to eight distinct lineages: (1) Chortis region of eastern Guatemala and western Honduras, (2) Pacific Mexico, (3) Gulf of Mexico, (4) Mayan region in Mexico and Guatemala, (5) Costa Rica, Honduras, and Nicaragua, (6) Ecuador, (7) western Panama, and (8) eastern Panama, Colombia, and Venezuela (Fig. 2). The uncorrected p-genetic distances among these lineages (Table 1) show values between 0.5% and 4.1% for 16S and between 2.1% and 6.5% for COI.

Morphology

The morphometric variation among lineages is reported in Table 2. For statistical analysis, we excluded the

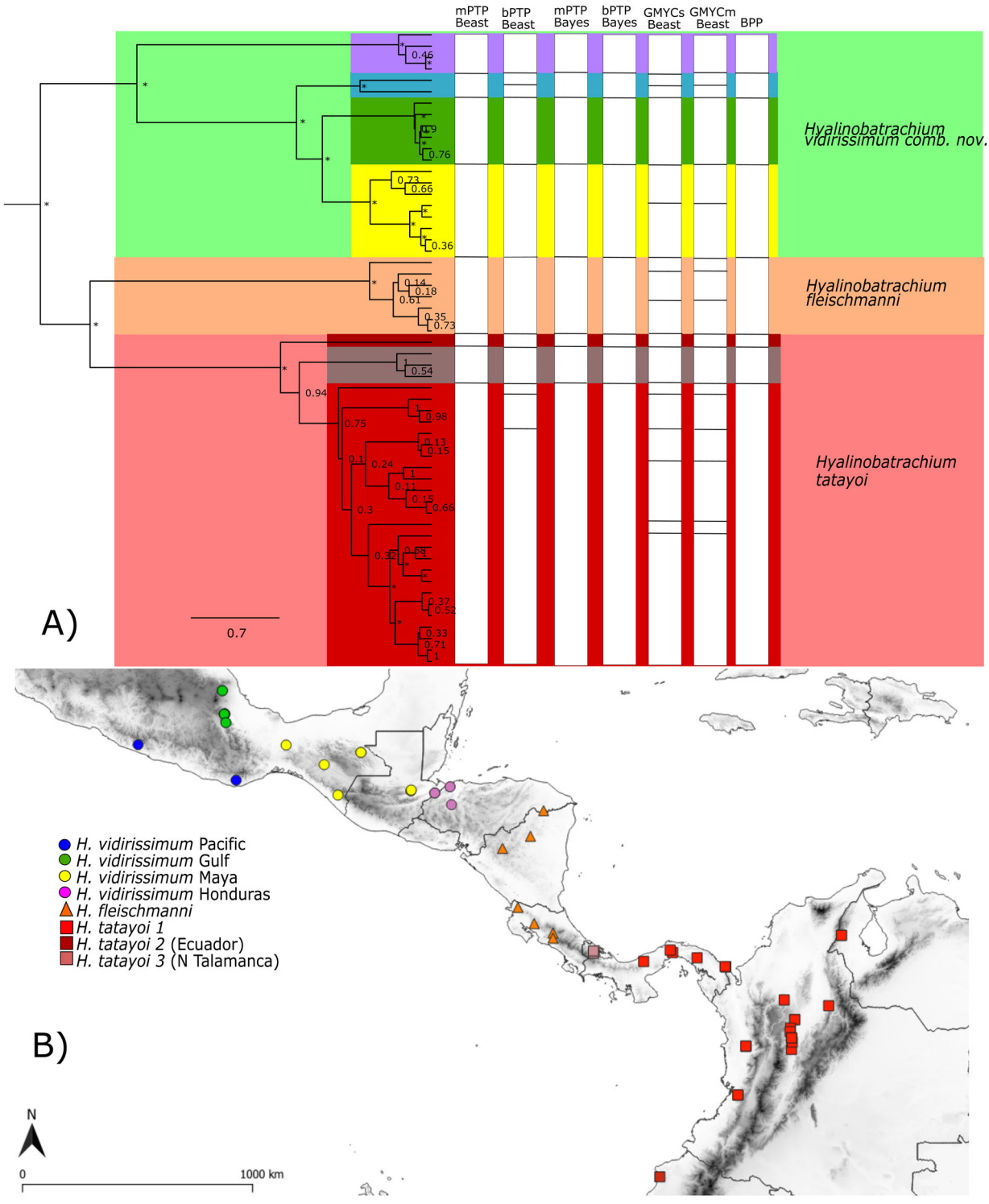

Fig. 2. (A) Ultrametric Bayesian reconstruction based on mitochondrial (16S, COI and ND1) and nuclear (POMC) sequences for *Hyalinobatrachium fleischmanni* species complex and results of molecular species delimitation by multiple approaches (GMYC multiple and single, mPTP, bPTP and BP&P). Asterisks on branches represent Bayesian posterior probabilities equal or greater than 0.95. Topologies were equal in MrBayes and BEAST. Scale bar below tree represents 0.7 million years. (B) Localization of the sequences included in molecular species delimitation analysis. Each shape represents the candidate species tested as in Figure 1.

Table 1. Pair of uncorrected p-distances for COI (upper) and 16S (lower) among the eight lineages gathered by molecular delimitation analysis.

	1	2	3	4	5	6	7	8
1- Gulf of Mexico	–	0.033	0.029	0.043	0.058	0.056	0.058	NA
2- Mayan region in Mexico and Guatemala	0.008	–	0.026	0.039	0.054	0.052	0.053	NA
3- Pacific Mexico	0.013	0.021	–	0.049	0.065	0.063	0.062	NA
4- Eastern Guatemala and Honduras	0.010	0.017	0.018	–	0.063	0.064	0.055	NA
5- Eastern Panama, Colombia and Venezuela	0.022	0.03	0.028	0.02	–	0.021	0.047	NA
6- Western Panama	0.028	0.036	0.034	0.026	0.007	–	0.041	NA
7- Costa Rica, eastern Honduras, and Nicaragua	0.03	0.033	0.041	0.03	0.021	0.027	–	NA
8- Ecuador	0.027	0.034	0.033	0.024	0.005	0.011	0.023	–

Table 2. Morphological measurements (in mm) of the lineages suggested by molecular delimitation (mean ± SD). * = metrics were not taken for the entire number of individuals. See Supplemental Material for statistical comparisons among groups.

			***H. viridissimum* comb. nov.**			
	H. fleischmanni	***H. tatayoi***	**Honduras**	**Gulf**	**Pacific**	**Maya**
N	19	85	1	28	23	12
Snout-vent length	21.67 ± 1.70	21.55 ± 1.57	21.09	23.67 ± 1.45	21.83 ± 1.03	24.11 ± 1.15
Head length	6.33 ± 0.71	6.20 ± 0.61	5.6	6.93 ± 0.56	6.21 ± 0.47	7.07 ± 0.83
Head width	8.41 ± 1.01	8.21 ± 0.63	7.77	8.97 ± 0.50	8.20 ± 0.53	9.08 ± 0.46
Interorbital distance*	3.88 ± 0.41	3.75 ± 0.50	3.66	3.98 ± 0.27	3.79 ± 0.24	4.27 ± 0.27
Eye length	2.28 ± 0.15	2.39 ± 0.29	2.48	2.38 ± 0.28	2.32 ± 0.15	2.37 ± 0.14
Eye nostril	1.86 ± 0.15	2.05 ± 0.34	1.98	2.52 ± 0.23	2.13 ± 0.31	2.48 ± 0.38
Finger III width*	1.24 ± 0.20	1.09 ± 0.16	1.16	1.25 ± 0.15	1.17 ± 0.13	1.10 ± 0.20
Femur length*	11.36 ± 0.51	12.05 ± 0.74	11.52	12.94 ± 0.79	12.40 ± 0.54	13.54 ± 0.53
Tibia length	12.08 ± 0.84	11.98 ± 0.76	11.22	12.96 ± 0.55	12.10 ± 0.75	13.34 ± 0.48

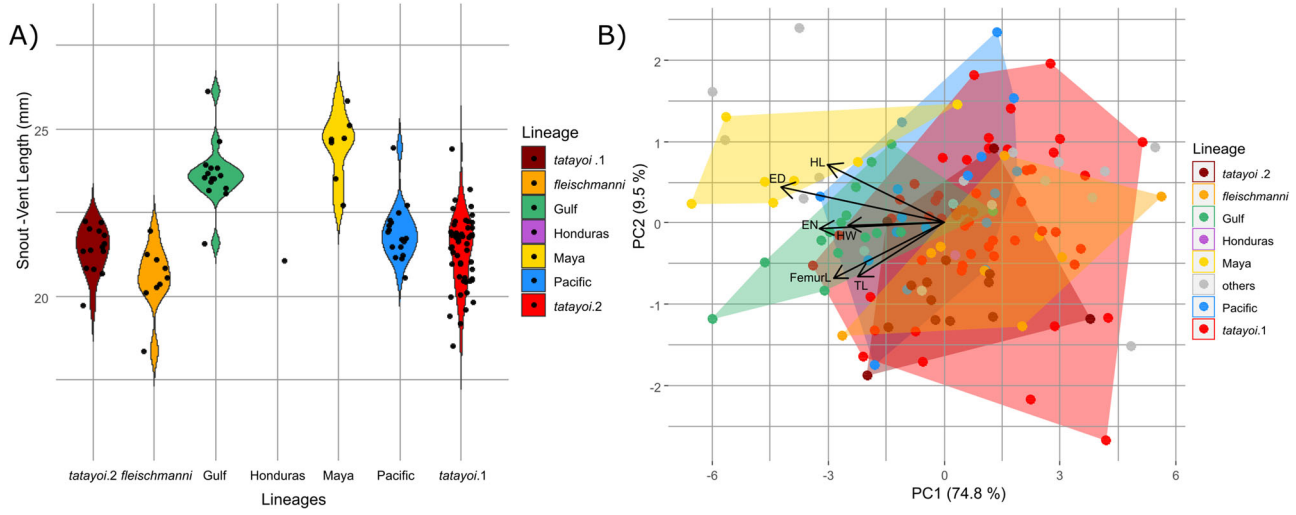

Fig. 3. (A) Violin plot of snout–vent length for males of candidate species, with Kruskal–Wallis test results per pair of lineages (statistical results are provided in Supplemental Material). (B) Principal component analyses of the residuals of the morphometric measurements against SVL for 132 males of *H. fleischmanni* complex plus other species within Hyalinobatrachium.

Chortis lineage due to the small sample size in this study (a single individual: AMNH-54777). The non-parametric tests show that males from the Mexican Gulf and Maya lineages have larger SVL compared with the other clades (Kruskal $Y = 48.055$, $P > 0.001$, $n = 115$, Fig. 3a). Similar results were encountered for the remaining metrics (Table S1). The morphospace generated by the two first components of the PCA with and without SVL correction showed no differences between the genetic groups (Fig. 3b). In both cases, the first component explains most (60.6% and 74.8%) of the variation and no metric was dominant in the contribution of the component. This absence of differentiation was also observed when including other known

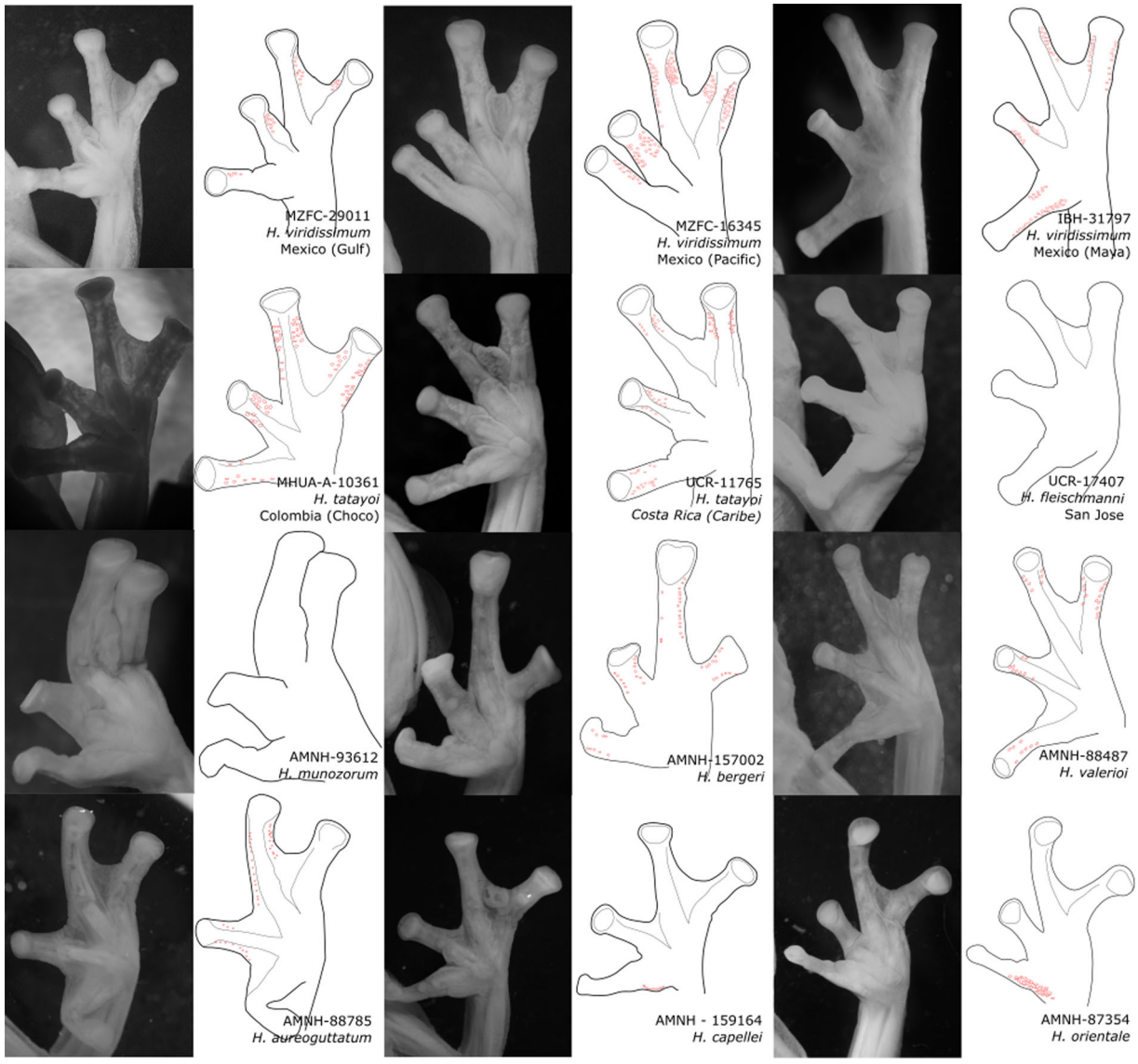

Fig. 4. Nuptial pads variation (or lack of) in some Hyalinobatrachium species. Type V forming glandular clusters in the webbing of all fingers in *H. viridissimum* **comb. nov.** (MZFC-25001, MZFC-16345, IBH-31797) and *H. tatayoi* (MHUA-A-10361, UCR-11765); absent in *H. fleischmanni* (UCR-17407) and *H. munozorum* (AMNH-93612); Type V as a discrete line in the webbing of all fingers in *H. bergeri* (AMNH-157002), *H. valerioi* (AMNH-88487), and *H. aureoguttatum* (AMNH-88785); a single line in thumb in *H. capellei* (AMNH-15916); type I-like in *H. orientale* (AMNH-87354).

Hyalinobatrachium species for the comparison (grey points).

Regarding categorical variables, the nuptial pads was the only character that shows enough variations among lineages. Males from Colombia, Ecuador, Venezuela, Panama, and the Caribbean region in Costa Rica show abundant warts on the webbing of hands (nuptial pads type V following Cisneros-Heredia & McDiarmid, 2007 categories) while males from San Jose (Costa Rica) lack warts (Fig. 4). Additionally, males from Mexican lineages show multiple states of warts within the same locality.

Bioacoustics

We obtained recordings for 64 individuals (Fig. 5). The pairwise comparisons using Wilcoxon rank sum test for each of the call variables according to the genetic clusters, showed significant differences of the Gulf and Mayan lineages with respect to the remaining lineages for the peak frequency (see Supplemental Material). There was also a significant difference in the duration of the call between the *fleischmanni* and *tatayoi* clades and among the three Mexican lineages. The frequency bandwidth showed significant differences between the lineages of the two big clades (Pacific, Gulf, and Maya

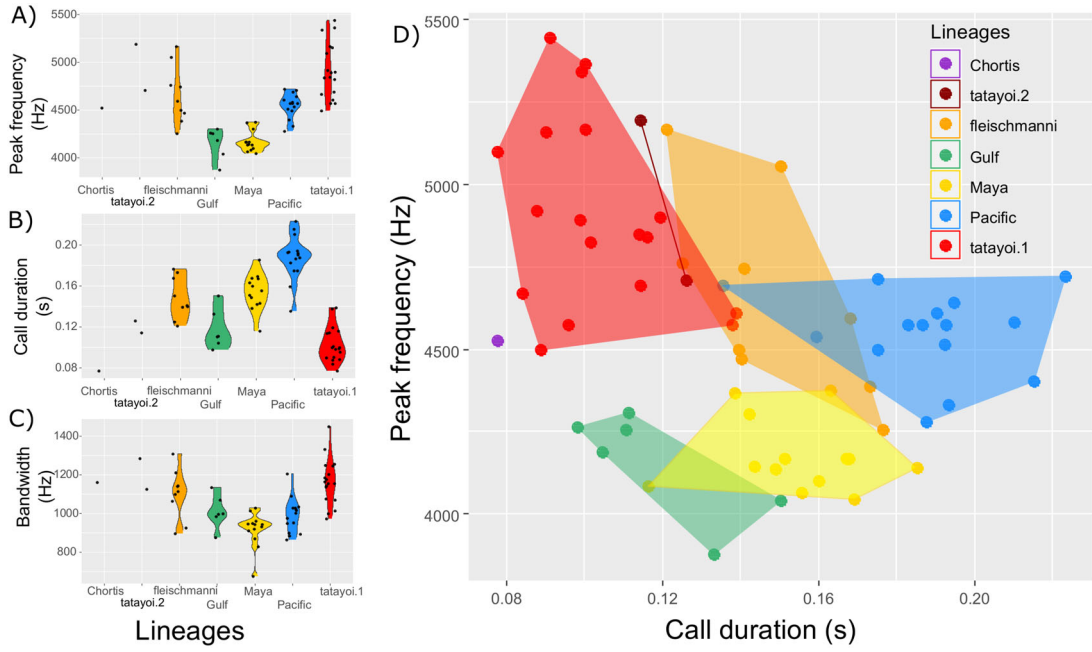

Fig. 5. (A–C) Violin plots of call variation per lineages for the three metrics analysed and (D) Acoustic space (in peak frequency vs. call duration) of the species hypothesized as hidden inside *H. fleischmanni*.

vs *fleischmanni* and *tatayoi*). In the visual inspection of acoustic space (Fig. 5D), there is an overlap between calls recorded for the two suggested lineages within *tatayoi* (*tatayoi.1* and *tatayoi.2*), calls from the Maya and Gulf lineages partially overlap, and calls from *tatayoi* and *fleischmanni* show marginal overlap in call duration. On the other hand, calls of the Pacific lineage and the single call obtained for the Chortis clade in Honduras do not overlap with any of the sister lineages (Maya and Gulf).

Taxonomic account

Based on the available evidence and following the consensus protocol for integrative taxonomy (Padial et al., 2010), the differences in molecular, bioacoustic, morphological and geographic information suggest at least three distinct species (Table 3). Therefore, we update the distribution range of two formerly described species (*H. fleischmanni* and *H. tatayoi*) and we resurrect a synonym for *H. fleischmanni* (*Hyalinobatrachium viridissimum* **comb. nov.**) to apply to the four lineages of the Northern clade. Additional information of the taxonomic conclusion is provided in the Discussion.

Species accounts

Hyalinobatrachium fleischmanni (Boettger, 1893)

Synonyms. *Hylella fleischmanni*: Boettger, 1893: 252; *Hylella chrysops*: Cope, 1894: 196**;** *Hyla fleischmanni*: Nieden, 1923: 225; *Centrolenella fleischmanni*: Noble, 1924: 69; *Centrolenella viridissima*: Taylor, 1942: 74; *Cochranella chrysops*: Taylor, 1951: 35; *Cochranella*: *fleischmanni* Taylor, 1951: 34; *Cochranella decorata*: Taylor, 1958: 50; *Cochranella millepunctata* Taylor, 1958: 53; *Cochranella fleishmanni* [*sic*]: Rivero, 1961: 153; *Centrolenella fleischmanni*: Goin, 1964: 1; *Hyalinobatrachium fleischmanni*: Ruiz-Carranza & Lynch, 1991, 24.

Holotype. Forschungsinstitut und Natur-Museum Senckenberg (SMF) 3760

Type locality. 'San José, [Cantón de San José, Provincia San José,] Costa Rica'.

Taxonomic history. *Hyalinobatrachium fleischmanni* was originally described by Boettger (1893) based on two syntypes from Canton de San José, San José Province, in Costa Rica, ~1,180 m asl (Mertens, 1967). The species was included in the *H. fleischmanni* group by Ruiz-Carranza and Lynch (1991). Starrett (1960) described the eggs and tadpole based on 16 specimens from San Jose de la Montaña, Heredia Province, Costa Rica. Savage (2002) redescribed the species including the information of the species biology, tadpole, and

Table 3. Multiple sources of evidence used in species delimitation for sister lineages inside the *H. fleischmanni* complex.

	Pairs of sister lineages			
Source of evidence	***fleischmanni / tatayoi***	***tatayoi* Colombia / Ecuador**	***viridissimum* comb. nov. Pacific / Honduras**	***viridissimum* comb. nov. Pacific / Maya**
Genetic p-distances (16S / COI)	2.1 % / 4.7 %	1.1 % / NA	1.8 % / 4.9 %	2.1 % / 2.6 %
Phylogeny-based species delimitation	delimited	delimited	delimited	delimited
Advertisement call differences	significant differences	no differences	differences (few data)	significant differences
Nuptial pads	two fixed states	one single state	unknown	highly variable
Morphometry	no differences	no differences	unknown	significant differences
Speciation event	yes	no	unknown	unknown

advertisement call description. He stated that *H. fleischmanni* is among the most studied Central American amphibians. Kubicki (2007) provides a description of the species, including a detailed range map for Costa Rica.

New data

Diagnosis. (1) Vomerine teeth absent; (2) snout rounded to semi-rounded in dorsal and truncated lateral profiles with region about nostrils slightly elevated, with a slight depression between them; (3) tympanum concealed, indistinct, lack of supratympanic fold; (4) dorsal skin shagreen; (5) ventral skin granular, transparent; (6) parietal peritoneum transparent, visceral and pericardium peritoneum covered by iridophores; (7) liver bulbous; (8) humeral spines absent; (9) hand webbing II 2+ — 3 III 2 — 2 IV, absent between fingers I and II; (10) webbing between toes I (I 1 — 2 II 1 — 2 III 1+ — 2- IV 2 1/2- — 1+ V; (11) ulnar and tarsal fold absent; (12) no nuptial pads in adult males; prepollex concealed; (13) finger I slightly larger than finger II; (14) disc of finger III width about 54% of eye diameter; (15) colour in life, dorsum lime green with yellow spots; colour of bones white; (16) colour in preservative, dorsum cream with black spots; (17) iris colouration in life golden to with dark reticulations; (18) dorsal surfaces of fingers and toes lacks melanophores; (19) males usually call below leaves (20); eggs greenish white, deposited on underside of leaves over streams as a monolayer in a laminar array; (23) snout–vent length (SVL) in males 21.7 mm ± 1.6 ($n = 15$), 26.1 females ($n = 1$).

Distribution and habitat. The species is widely distributed from 25 to 1,740 m asl in the Lowland Moist Forest, Lowland Wet Forest, Premontane Pine-Oak Forests, Premontane Wet Forest, and marginally into Lower Montane Wet Forest from the Mosquitia region of eastern Honduras and Nicaragua and southwards to the Tilarán range and Central Valley in Costa Rica (McCranie & Wilson, 2002; Sunyer et al., 2014). The species is ubiquitous and often common even in pastures and other cleared sites, where it occurs along small streams or areas with remnant riparian vegetation with an overstorey.

Advertisement call. Here we update the information of the advertisement call and the reproductive behaviour based on our results and former descriptions. The call consists of a single tonal note preceded by a short series of 3–7 pulses at a mean rate of 176.9 pulses/s (Fig. 1d). The overall call has a peak frequency of 4,659.6 ± 366.2 Hz and a duration of 148.3 ± 24.9 ms (N = 20). Males observed in Gaucimal River in Monteverde, Puntarenas and from San José (Costa Rica) call from the undersides of leaves from dusk (1800 h), with higher activity until 2100 h and decreasing until dawn (Gutiérrez-Vannucchi et al., 2019; Jacobson, 1985)

Comparisons. *Hyalinobatrachium fleischmanni* can be differentiated from most species of the genus by its visceral and pericardium peritoneum covered by iridophores. Among the species with the same condition (i.e., *H. tatayoi*, *H. viridissimum* **comb. nov.**, *H. bergeri*, *H. mondolfii*), *H. fleischmanni* differs by the lack of nuptial pads in males and by its advertisement call (Table 4) and by lack of white enamelled glands delimiting the jaw (presence of white enamelled glands delimiting the jaw in *H. viridissimum* **comb. nov.** and *H. tatayoi*). In particular, the peak frequency of *H. fleischmanni* is lower than those of *H. munozorum* which lacks nuptial pads too (4,659.6 ± 303.4 Hz and 5,011.5 ± 16.0 Hz respectively).

Conservation status. Even though the range of the species is now more restricted than before, the species can still be considered as Least Concern (LC) following IUCN (2017) categories, due to its wide distribution (more than 20,000 km^2) and high tolerance to disturbed habitats.

Table 4. Main acoustic parameters of the advertisement call of *H. fleischmanni* complex plus other closely related *Hyalinobatrachium* species (mean ± SD). Call data were obtained from the following sources: *H. carlesvilai* and *H. bergeri* (Castroviejo-Fisher et al., 2009), *H. mondolfii* and *H. kawense* Castroviejo-Fisher et al. (2011).

Species or lineage	Distribution	Call duration (s)	Peak frequency (Hz)
H. fleischmanni	Lowlands in Costa Rica and Nicaragua	0.148 ± 0.020	4659.6 ± 303.4
H. tatayoi	Widespread along Choco-Magdalena region and eastern slope of Merida range	0.105 ± 0.017	4916.2 ± 287.9
H. viridissimum **comb. nov.** Gulf	Veracruz state in Gulf of Mexico	0.118 ± 0.020	4154.5 ± 165.5
H. viridissimum **comb. nov.** Pacific	Pacific region of Madre del Sur, Mexico	0.188 ± 0.022	4549.5 ± 130.0
H. viridissimum **comb. nov.** Maya	South-eastern Chiapas of Mexico and Guatemala	0.155 ± 0.017	4174.0 ± 108.1
H. viridissimum **comb. nov.** Honduras	Nuclear Central America in Honduras	0.209	4048.2
H. carlesvilai	Amazonian Andean slopes of Peru and Bolivia	0.134 ± 0.013	4837.9 ± 85.8
H. mondolfii	Amazonas, Colombia and Pará, Brazil	0.190 ± 0.010	5011.5 ± 16.0
H. munozorum	Upper Amazon Basin in Ecuador, Colombia, and northern Bolivia	0.134 ± 0.027	5037.9 ± 244.0
H. kawense	French Guiana	0.090 ± 0.01	5285.5 ± 117.9
H. bergeri	Amazonian slopes of Andes in Peru and Bolivia	0.154 ± 0.019	4599.08 ± 69.9

Hyalinobatrachium tatayoi Castroviejo-Fisher et al., 2007

Holotype. Museo de Historia Natural La Salle (MHNLS) 17174

Type locality

'A stream near Tokuko (09°50′30.6″N, 72°49′13.6‴W; 301 m asl), Estado de Zulia, Venezuela.'

New data

Diagnosis. (1) Vomerine teeth absent; (2) snout semi-round to round in dorsal and truncate to round lateral profiles; (3) tympanum covered by skin; (4) dorsal skin shagreen; (5) ventral skin granular, transparent; (6) parietal peritoneum transparent, visceral and pericardium peritoneum covered in white iridophores; (7) liver bulbous; (8) humeral spines absent; (9) hand webbing II 2 — $3^{1/2}$ III 2 — 2 IV, absent between fingers I and II and basal between fingers II and III; (10) webbing between toes 1 — 2 II 1— 2 III 1^{-}— 2 IV 2 — 1 V; (11) enamelled ulnar and tarsal fold present; (12) nuptial pads in adult males; (13) finger I almost as long as finger II; (14) disc of finger III width about 56% of eye diameter; (15) colour in life, dorsum dark apple green with small pale yellow spots; colour of bones white; (16) colour in preservative, dorsum cream with purple melanophores; (17) iris colouration in life yellow with black flecks more concentrated towards the pupil creating a horizontal band that connects the pupil with the lateral edges of the eye; (18) dorsal surfaces of fingers lacks melanophores; small melanophores reaching the last phalange of the fifth toe; (19) males call above and below leaves (20); eggs deposited on underside of leaves over streams; (23) snout–vent length (SVL) in males 21.3 ± 1.4 mm, 21.9 ± 0.9 mm in females.

Distribution and habitat. *Hyalinobatrachium tatayoi* ranges from the Venezuelan Cordillera de Perijá, in the northern border between Colombia and Venezuela, through dry forests in middle and upper Magdalena valley including the xeric scrub ecoregion in Caribbean lowlands (Acosta-Galvis, 2012) up to the department of Tolima (Rada & Guayasamin, 2008), from the Isthmian-Atlantic moist forests (Caribbean slope of Talamanca range) in Panama and Costa Rica and from the Choco-Darien moist forests through Colombia to Esmeraldas province in Ecuador. The type locality is in submontane rainforest. The southernmost record to date is from the western Ecuador, at Cerro de Hayas (−02.7299, −79.6297, 127 m), ∼20 km south-west of Naranjal, province of Guayas, in fragments of riparian vegetation near paddocks and pastures (Cruz et al., 2017). The species ranges from sea level to 1,640 m asl.

Advertisement call. The call consists of a single note with a peak frequency of 4,916.2 ± 287.9 Hz ($n = 20$) and a duration of 104 ± 17 ms ($n = 20$). The first third of the call is pulsar composed by a series of 5–18 pulses at a mean rate of 258.6 pulses/s. The remaining two-thirds of the note are tonal (Fig. 1e-f). Calling activity began at dusk (1910 h) and decreased near midnight for Chocó and Magdalena populations in Colombia. In Ecuador, multiple males called from the upper surfaces of leaves at dusk, followed by a retreat to the undersides of the leaves after dark (Delia et al., 2010). During heavy rains, the species can be heard calling also between 0440 and 0530 h (L. Coyazos et al., unpubl. data).

Greer and Wells (1980) described the encounter and courtship calls based on individuals recorded in Barro Colorado (Panama). They described these calls in two

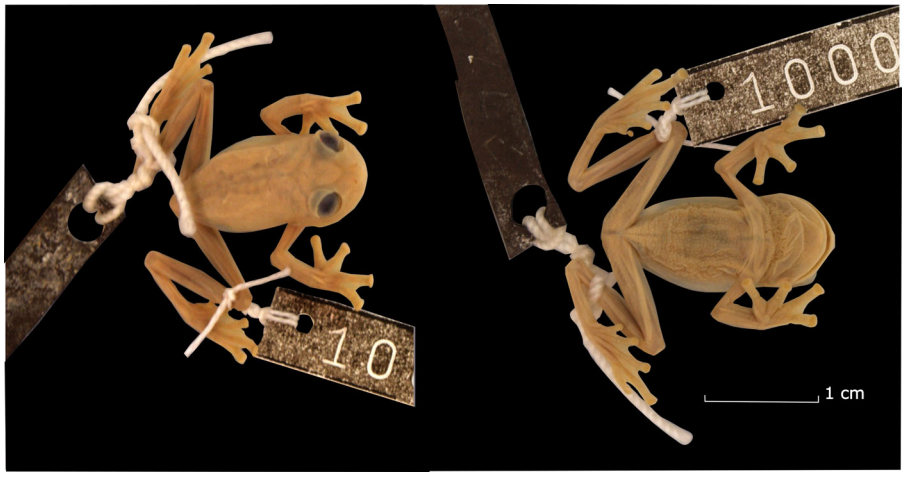

Fig. 6. Dorsal and ventral view of holotype of *Hyalinobatrachium viridissimum* **comb. nov.** (FMNH 100093). © Field Museum of Natural History. CC-BY-NC. a2dbe194-2421-472e-bb93-7290b2666a5b https://fm-digital-assets.fieldmuseum.org/426/161/C_viridissima100093d.jpg; https://fm-digitalassetsfieldmuseum.org/426/162/C_viridissima100093v.jpg (accessed 6 May 2019).

types: mews and chirps. Mew-type calls had a duration of 0.45 s and a range frequency between 4,238 and 4,852 Hz (during encounters) or a duration of 0.27 s and a range frequency between 4,194 and 4,546 Hz (during courtship). Chirp-type calls emitted during courtship had a duration of 0.10 s and a range frequency between 4,002 and 4,387 Hz.

Comparisons. *Hyalinobatrachium tatayoi* differs from most species of the genus by its visceral and pericardium peritoneum covered by iridophores. Among the species with the same condition (i.e., *H. viridissimum* **comb. nov.**, *H. bergeri*, *H. mondolfii*), *H. tatayoi* can be differentiated by its advertisement call, being shorter than all species except *H. kawense* (104 ± 17 ms for *H. tatayoi* and 0.090 ± 0.01 for *H. kawesne*, Table 4). *Hyalinobatrachium tatayoi* differs from *H. viridissimum* **comb. nov.** in the advertisement call, higher frequency (4,916.2 ± 287.9 Hz) in *H. tatayoi* (4,549.5 ± 130.0 Hz for *H. viridissimum* **comb. nov.**), and shorter duration 104 ± 17 ms in *H. tatayoi* (187.6 ± 21.6 ms for *H. viridissimum* **comb. nov.**). In this case, both species differs by the dorsal melanophores of two different sizes in *H. kawense* (small and minute), being absent or very small in *H. tatayoi*. Finally, the species differs from *H. fleischmanni*, *H. bergeri*, and *H. munozorum* by the presence of abundant nuptial pads type V in the hand webbing of males (*H. fleischmanni* and *H. munozorum* lack nuptial pads and those for *H. bergeri* form a single line; Fig. 4) and by the presence of white enamelled glands delimiting the jaw (white enamelled glands delimiting the jaw absent in *H. fleischmanni*).

Natural history. The species is found on leaves over small and big streams, calling in vegetation up to 10 metres above stream water, found also in the vegetation along big rivers such as the Atrato River in Choco (∼200 m width). The species is highly tolerant to water pollution, having been observed even downstream of mining camps. In Magdalena populations in La Dorada municipality (Caldas, Colombia), males were found during dry (February) and humid (May) seasons calling from the underside of leaves (Araceae and Heliconiaceae) and have been observed with up to three clutches. Eggs are predated by katydids and predatory wasps, but males show defensive behaviour by knocking the predator with their hind legs (Delia et al., 2010). Males show venter-to-venter combat which starts with both males dangling upside down while holding vegetation with their hind limbs and lasting for ∼20 minutes until one frog was knocked from the leaf (Delia et al., 2010). The embryonic development from cleavage to hatching was described by Salazar-Nicholls and del Pino (2015) based on samples from Ecuador.

Conservation status. The species could be considered as Least Concern (LC) following IUCN (2017) categories, due to its wide distribution and high tolerance to disturbed habitats. The species is found inside protected areas such as Katios National Park (Burbano et al., 2015) and Farallones de Cali National Park in Colombia, and in Barro Colorado Island National Monument, Panama (Greer & Wells, 1980).

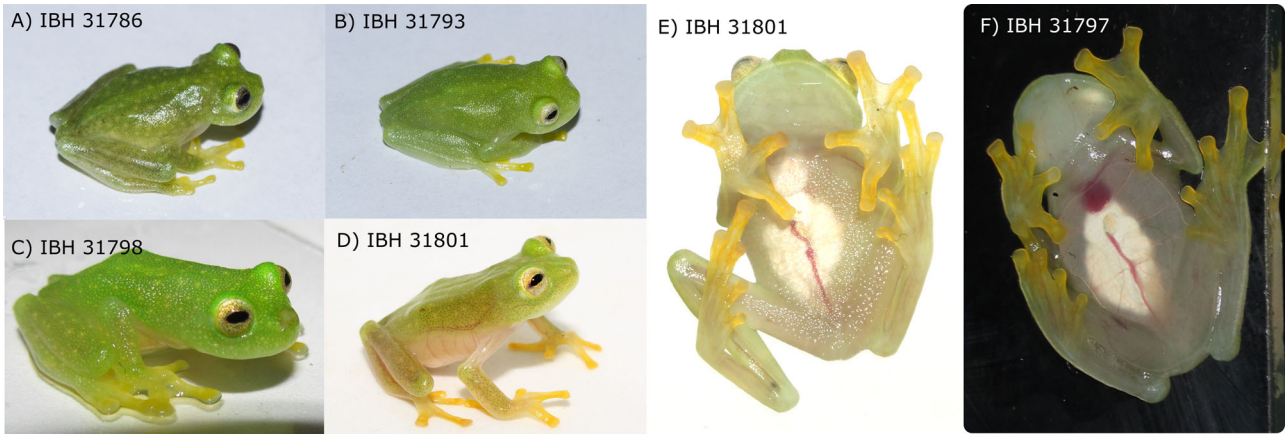

Fig. 7. Colour in life of *Hyalinobatrachium viridissimum* **comb. nov.** dorsal (A–D) and ventral (E–F), highlighting variation of iridophores in pericardium between populations.

Fig. 8. Detail of enamelled jaw and anal decoration of *Hyalinobatrachium viridissimum* **comb. nov.** (voucher MZFC-18408).

Hyalinobatrachium viridissimum **comb. nov.** (Taylor, 1942) (Figs 6, 7, 8)

Synonyms. *Centrolenella viridissima*: Taylor, 1942: 74; *Cochranella viridissima*: Taylor, 1951: 34.

Holotype. Edward H. Taylor–Hobart M. Smith collection (EHT-HMS) No. 27725, now Field Museum/FMNH 100093, Fig. 6), male, 2 August 1941, collected and described by Edward H. Taylor.

Type locality. Agua del Obispo, Guerrero, Mexico.

Generic placement. The species is placed in the genus *Hyalinobatrachium* (Ruiz-Carranza & Lynch, 1991, as modified by Guayasamin et al., 2009) on the basis of morphological and molecular data. The main diagnostic phenotypic traits of *Hyalinobatrachium* are: (1) ventral parietal peritoneum completely transparent; (2) digestive tract and bulbous liver covered by iridophores; (3) humeral spines absent; (4) white bones in life; and (5) males call from the undersides of leaves. All the aforementioned characteristics are shared by the species. Additionally, analyses of three mitochondrial genes place the species as a close relative of other *Hyalinobatrachium* species (Mendoza et al., 2019a); thus, generic placement in *Hyalinobatrachium* is unambiguous.

Diagnosis. (1) Vomerine teeth absent; (2) snout rounded to semi-rounded in dorsal and truncated lateral profiles with region about nostrils slightly elevated, with a slight depression between them; (3) tympanum concealed, indistinct, lack of supratympanic fold; (4) dorsal skin smooth; (5) ventral skin granular, transparent; (6) parietal peritoneum transparent, visceral and pericardium peritoneum covered by iridophores; (7) liver bulbous; (8) humeral spines absent; (9) hand webbing II 2+ — 31/2 III 2+ — 2- IV, absent between fingers I and II; (10) webbing between toes I 1- — 2 II 1- — 2 III 1— 3 IV 2+ — 1 V; (11) ulnar fold present, low; tarsal fold absent; (12) nuptial excrescences unpigmented between

hand webbing, Type V, prepollex concealed; (13) finger I slightly larger than finger II; (14) disc of finger III width about 51% of eye diameter; (15) colour in life, dorsum lime green with yellow spots; colour of bones white; (16) colour in preservative, dorsum cream to dark cream with black spots (in shape of asterisk and/or points) and white specks (sometimes the white sparks disappear) in visible areas of the body in rest position; (17) iris colouration in life golden to with dark flecks and reticulations and with a conspicuous silvery-white crescent-shaped mark; (18) dorsal surfaces of fingers and toes lacks melanophores; (19) males usually call below leaves; advertisement call consisting of single tonal note, with duration of 187.6 ± 21.6 ms and dominant frequency at 4,549.5 ± 130.0 Hz (20); eggs pale cream, deposited on underside of leaves over streams as a monolayer in a laminar array; (21) snout–vent length (SVL) in males 21.7 mm ± 1.6 ($n = 19$), 22.4 ± 0.6 on females ($n = 4$). The diagnosis refers to the Pacific lineage, associated with the type locality

Comparisons with other species. Most *Hyalinobatrachium viridissimum* **comb. nov.** can be differentiated from most species of the genus by its visceral and pericardium peritoneum covered by iridophores (except for some Mexican populations in Chiapas and Oaxaca with transparent peritoneum). Among the species with the same condition, *H. viridissimum* **comb. nov.** differs in having lower frequency (4,154.5 ± 165.5 Hz for populations from the Mexican Gulf and 4,549.5 ± 130.0 Hz for populations from Mexcian Pacific, Table 4). Also, *H. viridissimum* **comb. nov.** differs from *H. fleischmanni* and *H. munozorum* by the presence of Type V nuptial pads along the hand webbing (*H. fleischmanni* and *H. munozorum* lack nuptial pads).

Colour in life. Dorsum light green with yellow spots; concealed parts of limbs and ventral surfaces transparent; parietal peritoneum translucent, visceral peritoneum white and pericardium covered by iridophores (Fig. 7). Fingers and toes yellow. Jaw and folds in hand and feet and cloacal decoration enamelled (shiny white) (Fig. 8). Iris cream to yellow with dark points connected by a golden reticulation, being more intense near the horizontal pupil. The iridophores in pericardium are reduced or absent in some Mexican populations detected in San Gabriel Mixtepec, Oaxaca (Twomey et al., 2014).

Distribution. We consider *H. viridissimum* **comb. nov.** to be restricted to moist and cloud forest in the Pacific region in Madre del Sur subprovince (Morrone, 2017) in the Mexican states of Oaxaca and Guerrero from 20 to 1,275 m asl. We tentatively assign populations of the moist and cloud forests in Mexico in the states of Chiapas, Oaxaca, Veracruz, Puebla, and Tabasco, as well as those on both sides of the Motagua-Polochic-Jocotán fault system through lowlands of Guatemala and Honduras to this taxon, but further work is necessary to identify the species status of those populations.

Colour in preservative. Dorsum of head, body and limbs light cream to dark cream with black spots as points or small asterisks. Heart and all other visceral peritonea covered by white pericardium (Fig. 6).

Advertisement call. The call of Guerrero populations consists of a single and high-pitched tonal note with a peak frequency of 4,549.5 ± 130.0 Hz and a duration of 187.6 ± 21.6 ms without no major frequency modulation. The call shows a slight pulsar region in the first 28.1 ms (Fig. 1a) composed by a series of 1–6 pulses at a mean rate of 117.4 pulses/s ($n = 15$). Males usually call from underside of the leaves from 1 metre to more than 10 metres above water, although in a few cases they are observed calling from the upper-side of the leaves (Delia et al., 2010).

We also recorded the courtship call (Fig. 9) of a male (IBH-31806) from the underside of a Myrtaceae leaf at 2.5 m above the running water at 1940 h (before sunset) in San Vicente de Benitez municipality (Guerrero, Mexico). The call was emitted when another individual (likely a female) approached from the upper-side of the leaf. This call consists of 1–9 individual pulses followed sometimes by a long note, and it was emitted in call groups with short silent intervals of 536.3 ± 209.2 ms between calls. The courtship call shows a mean peak frequency of 4,441.4 Hz (± 88.8 Hz, $n = 88$), high variable duration (110.1 ± 47.5 ms, $n = 88$, $T^{\circ} = 17\,^{\circ}C$) and significant complexity and frequency modulation compared with the advertisement call.

Natural history. The species can be seen in vegetation along small and medium streams in conserved and disturbed habitats, including coffee plantations. Males can be heard calling from low to extremely high altitudes from the running water from dusk (1800–1900 h) and call rate goes down after midnight.

In Atoyac de Alvarez municipality (Guerrero), the species is syntopic with *Agalychnis moreletii* (Duméril, 1853), *Hypopachus variolosus* (Cope, 1866), *Tlalocohyla smithii* (Boulenger, 1902), *Leptodactylus melanonotus* (Hallowell, 1861), *Lithobates sierramadrensis* (Taylor, 1939), *Chaladrahyla pinorum* (Taylor, 1937), *Ptychohyla leonhardschultzei* (Ahl, 1934),

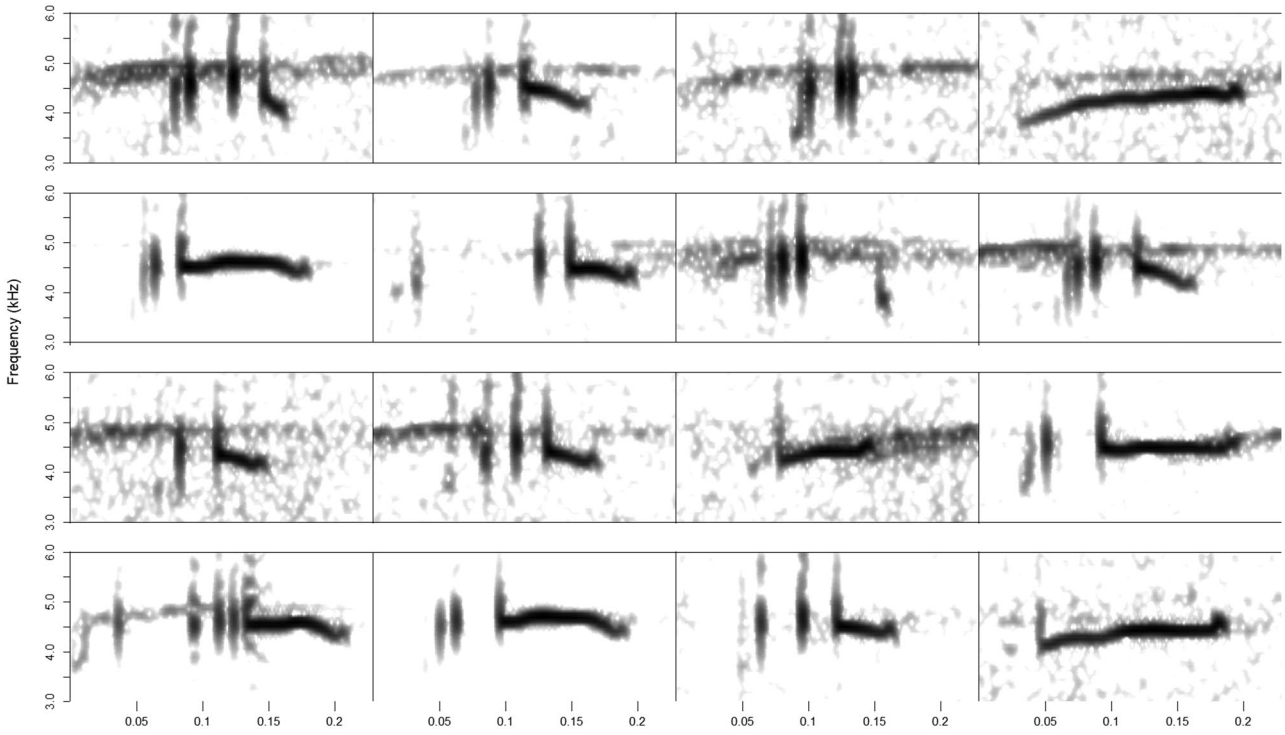

Fig. 9. Spectrograms of variation in courtship calls of *Hyalinobatrachium viridissimum* **comb. nov.** Voucher IBH-31806, T = 17 °C, SVL = 22.7 mm.

Rhinella horribilis (Wiegmann, 1833), and *Sarcohyla pentheter* (Adler, 1965).

Eggs are usually deposited in the underside of leaves above running water (Delia et al., 2013; Delia et al., 2010). Clutches have 22–23 pale-green eggs in a monolayer. The tadpole was described by Duellman and Tulecke (1960) based on individuals collected from the type locality in Agua del Obispo. The species shows parental care with brooding behaviour, males sleep next to the egg mass during the daytime and return to brood eggs three to five times in a single evening.

Etymology. The original name *viridissima* was adjusted to fit with the neutral gender of *Hyalinobatrachium* according to the Code (ICZN 1999). The name is derived from the Latin *viridis* meaning 'green' with the neutral superlative *-imum* to refer to 'the most' in reference to its bright green colour.

Conservation status. Considering that to date there is inadequate information to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status, the species could be considered as Data Deficient (DD) following IUCN (2017) categories.

Discussion

Neotropical anurans including glassfrogs frequently have high levels of cryptic diversity, and species richness is commonly underestimated (Fouquet et al., 2007; Funk et al., 2012; Guarnizo et al., 2015; Lyra et al., 2017; Paez-Vacas et al., 2019). The more frequent use of molecular data in taxonomy, plus DNA barcoding techniques, and automatized species delimitation analysis have increasingly revealed previously hidden species (Amador et al., 2018; Gehara et al., 2014; Ortega-Andrade et al., 2015). Unfortunately, some of these studies lack the integration of morphological, behavioural variation, and multilocus genetic data to most effectively test taxonomic hypotheses and to formally recognize cryptic species (Vences et al., 2013). In the past decade, the incorporation of molecular, morphological, and bioacoustic characters has been very useful to discover and describe new hidden species of glassfrogs for the Guiana Shield (Castroviejo-Fisher et al.,

2011), northern Peru (Twomey et al., 2014), and Colombia (Rada et al., 2019).

Our integrated study of species delimitation within the *H. fleischmanni* complex will be useful for future studies of the diversity and conservation of these frogs (Bickford et al., 2007; Funk et al., 2012). Similar to many amphibian species and in particular for *Hyalinobatrachium*, the species complex *H. fleischmanni* has been treated as a single species, due to its superficially uniform morphology across its distribution, even though Taylor & Tulecke (1960) found differences in tadpoles from Guerrero and Costa Rica, while Delia et al. (2010) found differences in the reproductive behaviour of Mexican and Ecuadorian lineages. Despite the apparent morphological conservatism in *H. fleischmanni*, here we reported differences on genetic, morphological, and acoustic characters.

Although molecular delimitation analysis has been used for the detection of cryptic species in amphibians and reptiles (e.g. Blair & Bryson Jr, 2017; Kuchta et al., 2016), it has been questioned when used as a definitive evidence of speciation processes since it assumes no population structure within lineages after speciation occurred (see Jackson et al., 2017; Sukumaran & Knowles, 2017). Therefore, molecular delimitation analysis tends to misidentify population structure as species boundaries. In populations with limited dispersal capacity or isolated by non-permeable barriers to gene flow, the phylogeographic structure is commonly pronounced (Avise, 2000; Hickerson et al., 2010), generating independence among populations (Hey & Pinho, 2012), sometimes congruent with phenotypic variation (Zamudio et al., 2016). Therefore, a rigorous interpretation of all sources of evidence is mandatory to identify if the differences detected by those methods actually correspond with speciation.

In our case, despite the fact that species delimitation analysis split *H. tatayoi* in three candidate species (Fig. 2), genetic distances with other populations were very low (16S = 0.8%, COI < 2.0%) compared with the candidate species thresholds suggested by Vences et al. (2005) (5% divergence for 16S and 10% for COI), Fouquet et al. (2007) (3% for 16S in Neotropical frogs), and Crawford et al. (2010) (8% for COI and 2% for 16S). As for molecular species delimitation, genetic distances alone must be used with caution, always with a geographic, phylogenetic, and integrative framework.

Furthermore, advertisement calls, morphometry, and nuptial pads of Ecuadorian populations show no differences regarding those from the remaining *H. tatayoi* populations. Therefore, we apply the name of *H. tatayoi* to all populations from Colombia, Venezuela, Panama, Ecuador, and Caribbean slope of Costa Rica.

On the other hand, we face a complex situation regarding divergence among allopatric lineages herein tentatively assigned to *H. viridissimum* **comb. nov.**. Molecular delimitation analysis suggested four lineages within the *H. viridissimum* **comb. nov.** clade, and bioacoustic analysis shows differences in acoustic space occupied by some of the genetic clusters (Fig 4.c). The most divergent populations (from the Chortis region, western Honduras, and eastern Guatemala) are separated from the remaining by the Motagua depression, an important geographic barrier delimiting distinct communities of vertebrates (Barrera-Guzmán et al., 2012; Ornelas et al., 2010; Rovito et al., 2015; Hofmann & Townsend, 2017). The remaining three lineages within the *H. viridissimum* clade show relatively low genetic distances (Table 1), even compared with values obtained in other sister glassfrog species (Amador et al., 2018; Rada et al., 2017; Twomey et al., 2014). We are in the process of acquiring additional morphological and acoustic data for Honduran specimens to evaluate the distinctiveness of this most-divergent lineage of the Northern clade. Until the status of Chortis populations can be resolved, the differences in call and morphology of the remaining inner clusters cannot be determined as products of rapid speciation events or structured populations showing local adaptation or divergence. Meanwhile, we suggest that the different populations of *H. viridissimum* **comb. nov.** at minimum constitute early stages of allopatric speciation and should be handled as independent evolutionarily significant units (ESUs) for conservation strategies (Moritz, 1994).

Populations formerly designated as *H. fleischmanni* in El Salvador have been registered in Cantón Montenegro, Metapán municipality, Santa Ana department (Köhler et al., 2005), in the Área Natural Rio Sapo, Arambala municipality, Morazán department (Henríquez & Greenbaum, 2014), and recently in the Area Natural Bosque de Cinquera in the Cabañas department (Segura et al., 2018). Due to its geographic location adjacent to both *H. fleischmanni* and *H. viridissimum* **comb. nov.**, it is necessary to obtain genetic, morphological, and/or bioacoustic information to compare with those species to determine to which clade these populations would be assigned.

The identity of *H. fleischmanni* sensu stricto was strongly supported for populations from east of Chortis highlands of Honduras to the Tilarán range and Central Valley of Costa Rica based on the uniformity of the morphological and bioacoustic traits (Figs 3 and 5), together with the absence of significant genetic isolation among populations (Fig. 2). Although the Talamanca range might be isolating *H. fleischmanni* and *H. tatayoi* populations (Mendoza et al., 2019a), considering the

wide distribution of both lineages in Costa Rica, it is necessary to perform an exhaustive revision of individuals and if possible some controlled reproductive experiments to identify if there are regions of secondary contact and hybridization between both species.

Further sampling at under-represented areas (i.e., Chortis region) is imperative in this case, and species delimitation by next-generation sequencing can provide robust phylogenies based on a massive number of independent loci. Speciation is not an instantaneous event and it ranges from continuous variation to population differentiation, ecotype formation, speciation, and post-speciation divergence (Nosil et al., 2009). Correct species delineation is fundamental to the discovery of life's diversity (Dayrat, 2005), but is not trivial given the biological, climatic, and geological changes and complexities such as those from Central American region (Bagley & Johnson, 2014). This study exemplifies how different lines of evidence can be integrated as a powerful tool to solve longstanding taxonomic problems and to discover cryptic lineages within diverse and taxonomically complex groups such as *Hyalinobatrachium*.

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No potential conflict of interest was reported by the authors.

Supplemental data

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