UNIVERSIDAD REGIONAL AMAZÓNICA IKIAM

Facultad de Ciencias de la Vida

Ingeniería en Ecosistemas

From the Atlantic forest to the Ecuadorian Andes: Phylogeny and biogeography of a new species of *Atopophrynus*, an *incertae sedis* lineage.

Grace Carolina Reyes Ortega

09 de abril del 2020, ciudad de Tena, Napo, Ecuador.

Ikiam
Universidad Regional Amazónica

Declaración de derecho de autor, autenticidad y responsabilidad

Tena, 26 de febrero de 2020

Yo, Grace Carolina Reyes Ortega con documento de identidad N° 1003637954, declaro que los resultados obtenidos en la investigación que presento en este documento final, previo a la obtención del título de Ingeniera en Ecosistemas son absolutamente inéditos, originales, auténticos y personales.

En virtud de lo cual, el contenido, criterios, opiniones, resultados, análisis, interpretaciones, conclusiones, recomendaciones y todos los demás aspectos vertidos en la presente investigación son de mi autoría y de mi absoluta responsabilidad.

Por la favorable atención a la presente, suscribo de usted,

Atentamente,

Firma:

Grace Reyes

_____

Grace Carolina Reyes Ortega

Ikiam
Universidad Regional Amazónica

Certificado de dirección de trabajo de integración curricular

Certificamos que el trabajo de integración curricular titulado: "From the Atlantic forest to the Ecuadorian Andes: Phylogeny and biogeography of a new species of *Atopophrynus*, an *incertae sedis* lineage.", en la modalidad de: artículo original, fue realizado por: Grace Carolina Reyes Ortega, bajo nuestra dirección.

El mismo ha sido revisado en su totalidad y analizado por la herramienta de verificación de similitud de contenido; por lo tanto, cumple con los requisitos teóricos, científicos, técnicos, metodológicos y legales establecidos por la Universidad Regional Amazónica Ikiam, para su entrega y defensa.

Tena, 26 de febrero de 2020

Firma:

Firmado electrónicamente por:
HUGO MAURICIO
ORTEGA ANDRADE

.....

Mauricio Ortega Andrade

C.I: 1717270811

Firma:

.....

Jennifer Guevara

C.I: 1500520869

Ikiam
Universidad Regional Amazónica

Dictamen de aceptación por parte del tribunal de grado

Yo Gabriel Massaine Moulatlet miembro del tribunal de grado del Trabajo de Integración Curricular denominado "From the Atlantic forest to the Ecuadorian Andes: Phylogeny and biogeography of a new species of Atopophrynus, an incertae sedis lineage..", elaborado por la estudiante Grace Carolina Reyes Ortega, declaro que el presente trabajo ha sido revisado y verificado, por lo que considero, conforme al Art. 12, literal 6 del Reglamento de Titulación de la Universidad Regional Amazónica Ikiam:

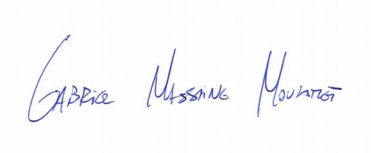
- Se acepta sin cambios: [x]

- Se acepta con cambios: [ ]

- No se acepta: [ ]

Observaciones:

En la ciudad de Tena , 13 de abril de 2020.



Gabriel Massaine Moulatlet
Miembro del Tribunal de Grado
Cédula: 1758617607

Ikiam
Universidad Regional Amazónica

Dictamen de aceptación por parte del tribunal de grado

Yo, PATRICIA ELENA SALERNO DOMINGUEZ, miembro del tribunal de grado del Trabajo de Integración Curricular denominado “MOLECULAR PHYLOGENY AND HISTORICAL BIOGEOGRAPHY OF AN INCERTAE SEDIS LINEAGE: A NEW SPECIES OF ATOPOPHRYNUS FROM THE ECUADORIAN ANDES" elaborado por la estudiante GRACE CAROLINA REYES ORTEGA, declaro que el presente trabajo ha sido revisado y verificado, por lo que considero, conforme al Art. 12, literal 6 del Reglamento de Titulación de la Universidad Regional Amazónica Ikiam:

- Se acepta sin cambios: [X]

- Se acepta con cambios: [ ]

- No se acepta: [ ]

Observaciones: Luego de la incorporación de cambios y correcciones, no tengo ningún comentario para agregar, más que felicitaciones por una muy buena tesis de pregrado a Grace.

En la ciudad de Tena, 14 de Abril de 2020.

Miembro del Tribunal de Grado
Cédula: 1759267857

Ikiam
Universidad Regional Amazónica

Dictamen de aceptación por parte del tribunal de grado

Nota: el texto en color gris es indicativo y debe eliminarse cuando se complete el formato. El documento estará debidamente legalizado con su firma en esfero azul

Yo **Liria Salazar, Jonathan** miembro del tribunal de grado del Trabajo de Integración Curricular denominado "**Molecular phylogeny and historical biogeography of an incertae sedis lineage: A new species of *Atopophrynus* from the Ecuadorian Andes.**", elaborado por el estudiante **Reyes Ortega, Grace Carolina**, declaro que el presente trabajo ha sido revisado y verificado, por lo que considero, conforme al Art. 12, literal 6 del Reglamento de Titulación de la Universidad Regional Amazónica Ikiam:

- Se acepta sin cambios: [X]

- Se acepta con cambios: [ ]

- No se acepta: [ ]

Observaciones: ____Las observaciones realizadas a la primera versión fueron atendidas, por lo cual considero que la estudiante puede solicitar fecha de sustentación en los lapsos establecidos en el nuestro Reglamento de Titulación._____

_____

_____

En la ciudad de Quito, 14 de abril de 2020

Jonathan Liria

Ph.D. Jonathan Liria
Miembro del Tribunal de Grado
Cédula: 1757595788

Acknowledgments

I am thankful to all the people and institutions that help me access specimens in the field and at the laboratory. To Universidad Regional Amazónica Ikiam for providing with human resources and the necessary laboratory work space to carry out this study. To H. Mauricio Ortega-Andrade Ph.D., Miguel Gómez Laporta M.Sc., Salomón Ramírez B.Sc., Raquel Betancourt M.Sc., Gabriel Vicente Gaona M.Sc. and undergraduate students at Ikiam, Jimmy Velasteguí, Michelle Guachamin, María José Sánchez and Pedro Chimbo for field assistance, collection of specimens, and the scientific collection management. To the "tigres", casual laborers of the Alto Tena and Atacapi communities in Tena, for their assistance with field work. To Andrea Carrera-Gonzalez M.Sc. for the guidance in the Molecular Biology Laboratory at Ikiam, as well as Katherine Apunte M.Sc. and Ikiam undergraduate students, Walter Quilumbaquin, Génesis Romero, Stalin Bermúdez, Sebastián Bermúdez, Jean Pierre Ramos, Jomira Yánez, Marilyn Suquillo and Mónica Sánchez, who participated in the molecular biology training process. To Dr. John D. Lynch for welcoming me into his laboratory and allowing me to review the scientific collection of Atopophrynus of the Instituto de Ciencias Naturales of the Universidad Nacional de Colombia, in Bogotá. To my advisors H. Mauricio Ortega-Andrade Ph.D. and Jennifer Guevara Ph.D., professors/researchers at Ikiam, for their direction, suggestions in the analyzes, and for reviewing this manuscript. To the Ministerio del Ambiente de Ecuador for the research and collection permits (Permit number: MAE – DNB – CM – 2017 – 0062). This work was supported by the project "On the quest of the golden fleece in Amazonia: The first herpetological DNA - barcoding expedition to

unexplored areas on the Napo watershed, Ecuador.", funded by the Secretaría Nacional de Ciencia y Tecnología del Ecuador, The World Academy of Sciences (TWAS), and the Red Lationamericana para el Estudio de la Biogeografía Neotropical (Red-Bion). I also thank the comments from the Graduate Tribunal members.

General index

Tables index

Figures index

Supporting information index

Resumen

La historia geológica de América del Sur ha impulsado la diversificación de especies neotropicales, a través de eventos de dispersión, vicarianza y extinciones como resultado de los cambios en la orogenia de la región. Un grupo neotropical que responde a los cambios geológicos relacionados con la elevación de los Andes son los anfibios (Anura). En este estudio, se discute la historia biogeográfica de *Atopophrynus*, considerado como *incertae sedis* dentro de Brachycephaloidea, además de describir una nueva especie de los Andes en Ecuador en base a en evidencia genética y morfológica. Se recuperó la posición filogenética de *Atopophrynus* usando ADN mitocondrial y nuclear, a través de análisis bayesiano y de máxima verosimilitud. Adicionalmente, el tiempo de divergencia de los taxones hermanos incluye la información calibrada en tiempo geológico, para contrastar el papel de la orogenia de América del Sur sobre su diversificación en los últimos 65 Ma. En un contexto más amplio, se analizó la historia biogeográfica usando una reconstrucción de áreas ancestrales por áreas biogeográficas, incorporando un análisis estadístico de dispersión y vicarianza. Los análisis filogenéticos revelan que *Atopophrynus* está relacionado con *Crossodactylus*, perteneciente a la familia Hylodidae, un grupo distribuido a lo largo del bosque Atlántico sur oriental de Brasil. La monofilia de Hylodidae no está respaldada por los resultados, por lo que es necesario incorporar información adicional para comprender las relaciones filogenéticas y la taxonomía del grupo. De manera interesante, la nueva especie es parte de uno de los clados más antiguos de Anura, con un origen sudamericano que se remonta a aproximadamente 33,48 millones de años. Se sugiere que los cambios paleogeológicos en América del Sur

promovieron su especiación, donde se identifican dos eventos de dispersión y vicarianza desde el Escudo Brasileño hasta el noroeste de los Andes, en el Paleógeno.

Palabras clave: Anura, biogeografía histórica, cronograma, sistemática, Hylodidae.

Abstract

The geological history of South America has driven the diversification of Neotropical species through dispersal, vicariance, and extinction events as a result of changes in the orogeny of the region. A Neotropical group that responds to geological changes linked to the Andes uplift are the amphibians (Anura). Herein, I discuss the biogeographical history of the amphibian genus *Atopophrynus*, considered as *incertae sedis* within Brachycephaloidea, with the description of a new species from the Ecuadorian Andes based on genetic and morphological evidence. I recovered the molecular phylogenetic placement of *Atopophrynus* using mitochondrial and nuclear DNA, through Bayesian and Maximum likelihood analyses. In order to explore the role of the South American orogeny in the diversification of my study group in the last 65 Ma I used divergence times from sister taxa that incorporates information calibrated in geological time. In a broader context, I analyzed the biogeographical history using an ancestral area approach, incorporating a statistical dispersal and vicariance analysis (S-DIVA). Phylogenetic analyses reveal that *Atopophrynus* is related to *Crossodactylus,* belonging to the family Hylodidae, a group distributed along the southeastern Atlantic forest in Brazil. The monophyly of Hylodidae is not supported by our results, thus being necessary to incorporate additional information to understand the phylogenetic relationships and taxonomy of the group. Interestingly, the new species (*Atopophrynus ikiam* sp. nov.) is part of one of the most ancient clades within Anura, with a South American origin dating back to approximately 33.48 Mya. I show evidence that the paleogeological changes in South America promoted its speciation, with two potential

episodes of dispersion and vicariance from the Brazilian Shield to the Northeastern Andes regions in the Paleogene.

Key words: Anura, historical biogeography, chronogram, systematics, Hylodidae.

Introduction

It is widely acknowledged that biodiversity is not distributed homogeneously across the Earth, with many taxa rather exhibiting clear broad-scale patterns in their spatial distribution [1–3]. A particularly well-documented pattern is that, for many plants and animals, the highest species richness is concentrated in tropical regions [1–6]. Among the different mechanisms that simultaneously drive such pattern and potentially explain why the tropics are highly speciose are major geological events that have intervened in the dispersal and isolation of populations [3,7]. The main evidence comes from the geological history of areas considered as biodiversity hotspots, such as the rising of the Andes in South America [7,8], Western Ghats in India [9,10], and several other mountain ranges in the world [8,11]. These geological events have had direct and indirect implications in generating favorable conditions for the speciation of various groups [12–19].

Interestingly, South America tops the list of biodiversity hotspots, including the Tropical Andes, Brazil´s Atlantic Forest, Chocó/Darién/Western Ecuador, Brazil´s Cerrado, and Central Chile, which are all considered high priority areas for conservation [8,11]. The geological history of South America reveals a gradual elevation of the Andes from south to north with the formation of some key Amazonian systems, such as Sub-Andean river, Pebas wetland, Acre system, and the Andean nutrient supply to the eastern Amazonia [7,20,21]. A number of studies have provided compelling evidence that the change in the Amazonian orogeny through geological time has played key roles on the speciation patterns of various taxa [22–28]. In birds, for example, the chronological elevation from the southern central

Andes to the northern Andes and the formation of the drainage system in Amazonia were decisive in the diversification of several lineages [22,23]. Similarly, the Andean uplift also appears to have driven the evolution and diversity of many arthropods [24–26]. For instance, in Neotropical butterflies, there is evidence that the Amazonian Pebas favored diversification of Andean species, which later dispersed to the Amazonia following the disappearance of the Pebas system [25]. Diversification of plants species, belonging to coffee family (Rubiaceae), may have also occurred when their distribution was engulfed between the North and Central Andes by a geographic barrier (the "Western Andean Portal"), with dispersal taking place towards the southern Andes only after the barrier disappeared; however, for Rubiaceae, speciation and dispersal between the Andes and Amazonia may have also been restricted by the presence of the Pebas system [27]. Similarly, a biogeographic analysis reveals that the uplift of the Andes in the Neogene allowed the diversification of wax palms (genus *Ceroxylon*) through processes of geographical migration from south to north [28]. While the association between geological history and diversification patterns of a vast number of plants and animals is increasingly well documented, understanding why and how these patterns have emerged across different taxa in highly biodiverse regions constitutes one of the most significant intellectual challenges in biogeography and evolutionary ecology.

By integrating phylogenetic and ancestral area reconstruction analyses, the major geological events in South America have been shown to drive the diversification and biogeographical history of several groups of Neotropical amphibians [29–37]. The species richness patterns of several groups of amphibians are associated with the geological time of

the Andes, one of the most biodiverse regions in South America [29]. For instance, evidence suggests that the species richness exhibited by poison dart frogs (Dendrobatidae) may be the result of several migrations with a radiation of 10 mya, congruent with the uplift of the Andes, a geological event regarded as the main source of dispersal of this group in the Amazonia [30]. Likewise, the divergence of glass frogs, Allocentroleniae (Centrolenidae + Allophrynidae), is congruent with the initial rising of the Andes dating back to the early Oligocene and Miocene, thus suggesting the Andes as a major center of diversification, but also as the original source of diversity of the Allocentroleniae clade [31]. In the case of the marine or giant toads (Bufonidae: *Rhinella marina* group), the complex is resolved with the divergence of several clades dating back to the Miocene, with evidence suggesting that geological events such as the Pebas system in the Amazon basin and the uplift of the Andes intervened in their diversification and historical biogeography [32,33]. Other reconstructions suggest that some groups, such as the Phyzelaphryninae (*Adelophryne* + *Phyzelaphryne*) and the genus *Adenomera* (Leptodactylidae), originated in the Amazonia and dispersed to the Atlantic forest during the early Miocene [34,35]. On the other hand, frogs of the genus *Eleutherodactylus* (Anura: Eleutherodactylidae) may have undergone three independent dispersal events, with two ancestors that dispersed to northern Central America from South America in the early Paleocene and from the Caribbean at the end of the Eocene, and several other dispersal events from South America related with the Isthmus of Panama in the Pliocene [36]. In the case of the direct-developing frogs (Craugastoridae: *Pristimantis*), evidence suggests the Northwestern Andes of Colombia and Ecuador as the most important

region for origin and diversification of the group [37]. Thus, there is strong existing evidence that the paleogeology of the South America Shield has promoted the diversification of several amphibian species, with a dynamic connection between the Andes uplift and Amazon basin. Despite this, our knowledge of the diversification patterns as a consequence of major geological events in areas of high diversity in South America is still limited for many other amphibian lineages, thus requiring additional data and analyses.

Atopophrynus Lynch and Ruiz-Carranza, 1982 is a monotypic genus considered as *incertae sedis* within the superfamily Brachycephaloidea, but without being assigned to any specific family [38]. Its phylogenetic position has remained unresolved within the most up to date phylogenies of amphibians [39–41]. Early studies originally assigned it to the family Dendrobatidae based on descriptions of the small Andean frog *Atopophrynus syntomopus* Lynch and Ruiz-Carranza, 1982 [42]. However, the descriptions considered only three known specimens from the top of the Cordillera Central in Antioquia, Colombia [42]. The genus was later re-described and assigned (*sensu lato)* to the family Leptodactylidae, with a possible relationship to *Geobatrachus* [43]. The assignment within Leptodactylidae, however, has been regarded as provisional because the taxonomic position, based on morphological characters, also classifies it within the family Bufonidae [43]. For more than two decades, the taxonomic position of *Atopophrynus* remained uncertain until it was provisionally assigned to Strabomantidae [44], recognizing five genera within the family: *Atopophrynus* + *Dischidodactylus* + *Euparkerella* + *Geobatrachus* + *Niceforonia*. The assignment to Strabomantidae was based on morphological reviews and on previous studies, but did not

include new specimens [42,43]. Thus, the inclusion of *Atopophrynus* in Strabomantidae continues to be debated as it was done without molecular analyses. The resolution of the phylogenetic position of *Atopophrynus* is therefore considered as pending [38]. However, beyond resolving its phylogenetic position, this study becomes relevant in a biogeographical approach [45–47], which has not been analyzed yet. In particular, it is interesting to address the speciation events of *Atopophrynus* and related lineages, and how these may relate to the geological history of the Neotropical region in South America.

Interestingly, scenarios where the Pan-Amazonia extended over most of northern South America (65 to 33 Ma) [7] could have provided appropriate conditions for the migration and dispersal of ancestral lineages. This geological time scenario is congruent with the diversification of Hyloidea, one of the three major clades that comprise ~88% of extant anuran species [14]. Later, the populations that colonized the western Amazonia would be expected to be isolated by geographic barriers, such as the initial northwestern Andes uplift [7]. In the case of the direct-developing frogs, the northwestern Andes in Colombia and Ecuador was the only region present in the Early Oligocene (35-23 Mya), which played an important role in the diversification of the group [37]. However, more current scenarios involving the uplift of the northern Andes and gradual expansion of the *terra firme* rainforest (10 to 2.5 Mya) [7] were also important in the diversification of recent lineages, thus are increasingly considered as centers of diversification of Andean frogs [29–31]. In this study, I describe a new species of *Atopophrynus* from the northeastern slopes of the Andes in Ecuador, and use a combination of genetic and morphological data to resolve its *incertae*

sedis status. Additionally, I analyze the phylogenetic relationships and speciation patterns of the new species to test hypotheses related with the paleogeology of the Andes uplift and Amazon basin. In this context, I addressed the following research questions: Is the new species related to ancient or recent Anuran lineages?, How does the origin of new species relate to major geological events of South America?.

Methods

Study area and sampling

Specimen collection took place at the Reserva Biológica Colonso Chalupas in the northeastern slopes of the Ecuadorian Andes. A total of five specimens potentially belonging to the new species were collected at a site characterized by montane evergreen forest habitat, located at an elevation of 2239 m (0.938135°S, 77.94898°W). Fieldwork was conducted during 17-21 December 2016 and 15-18 November 2018. The specimens were euthanized by the addition of lidocaine (3%), and then liver tissue samples were extracted in the field and preserved in 96% ethanol. The whole specimens were fixed with 96% ethanol and stored in 70% ethanol and deposited at the collection of the Integrative Biology Laboratory at the Universidad Regional Amazónica Ikiam. Taxonomic identification of the specimens was done by comparisons with the type series of *Atopophrynus syntomopus* from Colombia as well as published literature [42,44]. Permits for specimen collection was provided by the Ministerio del Ambiente de Ecuador (No. MAE – DNB – CM – 2017 – 0062).

Morphological characteristics

To compare the new species, the holotype of the monotypic species *A. syntomops* (ICN 8611) was reviewed at the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá. It is one of three specimens collected at the Cordillera Central, Departamento Antioquia, Colombia, at 2780 m altitude. Diagnosis, characters, and description of the new species followed the terminology for Strabomantidae frogs [48]. Photographs of the individuals were taken under an Amscope 3.5X-90X Trinocular LED Boom Stand Stereo Microscope and measurements were taken with a dial caliper (0.02 mm precision). Fifteen morphological measurements most commonly included in anuran species descriptions were registered [49]: snout-vent length (SVL) = distance from tip of snout to posterior margin of vent, head width (HW) = largest width of head distance at level of jaws, head length (HL) = distance from the tip of the snout to posterior of jaws, horizontal eye diameter (ED) = horizontal distance from the anterior and posterior corner of the eye, inter-orbital distance (IOD) = shortest distance between the anterior corners of the orbits, internarial distance (IND) = shortest distance between the inner margins of nostrils, eye-nostril distance (EN) = distance from anterior margin of eye to the posterior margin of the nostril, snout length (SL) = distance from the tip of the snout to the anterior margin of the eye, width of upper eyelid (UEW) = greatest width of the upper eyelid margins, snout-nostril length (NS) = distance from the center of the external nostril to the tip of the snout, hand length (HAL) = distance from the base of the outer palmar tubercle to the tip of finger IV,

thigh length (THL) = distance of thigh from the vent to the knee, tibia length (TL) = distance from the outer surface of flexed knee to the heel, foot length (FL) = distance from the base of the inner metatarsal tubercle to the tip of toe IV, toe IV disk width (Toe4DW) = greatest width between the edges of toe IV disk. I determined the sex of specimens by direct inspection of the gonads.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from animal tissue using the Isolation of Genomic DNA protocol from the Wizard Genomic DNA Purification Kit [50,51]. Tissue samples were first prepared by crushing to lyse nuclei. Next, the lysis and protein precipitation solutions were added, and finally, the DNA precipitation and rehydration solutions were used to preserve the DNA extracted.

Two mitochondrial genes (12S rRNA and 16S rRNA) and one nuclear gene (Recombination-activating gene 1 RAG-1) were amplified using the Polymerase Chain Reaction [52]. The information about primers used for amplification and their corresponding sequences are listed in Table 1. Each PCR reaction per sample was composed of a 25 µl reaction mix containing: 12.5 µl GoTaq Green Master Mix, 4.5 µl H_20, 1.5 µl on 10 µM of Forward and Reverse primers, and 5 µl of purified DNA. Amplification was performed on an Applied Biosystems GeneAmp PCR System 9700 thermal cycler. The amplification program was set with an initial denaturation of 95°C (5 min) followed by 35 cycles of 95°C (30 sec), 57°C (30 sec), 72°C (15 sec), with a final extension temperature of 72°C (5 min) and 4°C for an

unlimited period of time. Amplified DNA products were visualized by electrophoresis on a 2.5% agarose gel and post-staining with Tris/Borate/EDTA buffer (TBE) under blue light. PCR-amplified sequences were purified using illustra™ ExoProStar™ Enzymatic PCR and Sequencing Clean-Up Kit. Sequencing was performed in both DNA strain directions, and the procedure was undertaken by Macrogen services, Seoul, South Korea (http://www.macrogen.com).

Phylogenetic analyses

The chromatographs resulting from sequencing were revised and edited using Geneious Prime v.2020.0.5 software [53,54]. The amplified sequences for each gene were compared and identified on the basis of similarity to those in GenBank database [55] by using BLAST [56]. Then, the sequences with the highest percent of identity (82.04 - 90.69%) were downloaded and aligned with the sequences of the new species. Additional sequences of other species of the Hyloidea lineage (Anura) were considered to reconstruct the phylogenetic relationships of the new species. Sequences for 115 taxa corresponding to ingroup taxa were obtained from GenBank [55] and aligned with sequences generated for the new species. Outgroups included were *Bombina variegata*, *Discoglossus montalentii*, *Alytes obstetricans,* and *Uperoleia laevigata*. Sequences were aligned and visualized using Geneious Prime v.2020.0.5 [53,54]. The alignment type was “Global alignment with free end gaps” with a cost matrix of 65% similarity. The large and small subunits of the 12S and 16S mitochondrial ribosomal genes were edited in the alignment result, on hypervariable regions.

All sequences used in this study are specified in Table 2.

A concatenated genetic matrix was constructed with 12S, 16S, and RAG-1 genes in Mesquite v.3.2 software [57]. The best nucleotide evolution models were evaluated for non-coding genes (12S and 16S) and for three partitions by codon in the codifying gene (RAG1) with PartitionFinder2 [58] in CIPRES Science Gateway v. 3.3 [59]. The GTR + I + G was the best nucleotide substitution model for all partitions. Phylogenetic analyses were performed using Bayesian Inference (BI) and Maximum Likelihood (ML) methods on the CIPRES portal. Phylogenetic Bayesian Inference was performed with MrBayes v.3.2.6 [60], with the following settings: two simultaneous runs, four Markov chain Monte Carlo [MCMC], and 50 000 000 generations per run. The burn-in parameter discarded the 25% of generations from each run. Stationarity and convergence of MCMC runs were statistically assessed in Tracer v1.6 [61]. Phylogenetic analyses using ML were conducted in Garli on XSEDE v2.01 [62], using the same settings described previously above for BI. Tree support was evaluated using ML bootstrapping algorithm with 100 bootstraps configuration. Finally, the phylogenetic and time trees were edited using FigTree v1.4.2.

Time tree and biogeographic reconstruction

To estimate divergence times for the new species and its relatives, a calibrated time tree was reconstructed with BEAST v2.5.1 [63]. The configuration XML file was created in the Beauti v2.5.1 software following the settings specified in the Beast v2 manual [64,65]. The site models were linked by mitochondrial and nuclear genes partitions, while the clock model

and trees were linked for all partitions. The site model was set as GTR + I + G with interchangeable nucleotide frequencies estimated. A relaxed clock log normal model was set, considering a Yule model for the tree priors. Five secondary calibration points were used as secondary information provided in a time tree for Nobleobatrachian frogs [39]. The nodes were *Melanophryniscus*, *Rhinella*, *Duttaphrynus*, *Anaxyrus* and *Incilius* 48.7 (67.1-34.0) Mya, *Engystomops* and *Pleurodema* 39.9 (56.3-27.0) Mya, *Nymphargus*, *Centrolene*, *Cochranella*, *Hyalinobatrachium* and *Allophryne* 46.3 (65.1-31.5) Mya, *Stefania*, *Gastrotheca*, *Cryptobatrachus* and *Hemiphractus* 55.2 (75.4-38.8) Mya, and *Phrynopus*, *Bryophryne*, *Oreobates*, *Lynchius*, *Pristimantis*, *Microkayla*, *Ischnocnema*, *Brachycephalus*, *Eleutherodactylus*, *Diasporus*, and *Adelophryne* 54.2 (73.9-38.4) Mya. Analyses were run for 150 000 000 generations with sampling every 1000 steps. The runs outputs were visualized in Tracer v1.6 [66] to confirm the stationarity and convergence of the posterior distribution of model parameters emphasized on ESS values > 200. The consensus tree was performed with TreeAnnotator v2.5.1 software with the burn-in set to 15 000 generations.

To reconstruct ancestral biogeographical areas, all the trees generated in BEAST v2.5.1 and the consensus tree were used as input to perform a statistical dispersal-vicariance analysis (S-DIVA) [67], implemented in RASP v.3.02 software [68]. The regionalization of areas for South America follows the division used to reconstruct the biogeography of Dendrobatidae [30]: (A) Regions outside South America, related for outgroups; (B) Guiana Shield, (C) Amazon Basin, (D) Venezuelan Highlands, (E) North Eastern Andes, (F) North Western Andes, (G) Central Eastern Andes, (H) Central Western Andes, (I) Chocoan

rainforest, (J) Central America, (K) Brazilian Shield, (L) Austral Region, (M) Wide distribution (more than 3 regions). The information about the distribution of each species was obtained from the Amphibian Web Database [69]. Nearly 20% of trees were discarded, and a random sample of 100 trees was selected as input for statistical estimations of support for ancestral areas. The analysis was set to allow a maximum number of three combinations among all biogeographical regions. This setting allows generation of the most likely ancestral areas model and provides information about vicariance, dispersal, and extinction events [67].

Results

Species description

Atopophrynus ikiam sp. nov.

Holotype. HMOA 2040 (Fig 1), an adult female collected at the Reserva Biológica Colonso Chalupas (RBCC; Fig 2A), 0.93762°S, 77.94898°W, 2196 m altitude, Tena, Napo province, Republic of Ecuador, on 20 Dec 2016 by H. Mauricio Ortega-Andrade, Grace C. Reyes-Ortega, Michelle Guachamin, and Jimmy Velasteguí.

Paratype. Three females and one male (HMOA 2055, HMOA 2165, HMOA 2166, and HMOA 2168), collected from the same locality of holotype. HMOA 2055 was collected at RBCC, 0.93687°S, 77.94996°W, 2219 m altitude, on 21 Dec 2016 by H. Mauricio Ortega-Andrade, Grace C. Reyes-Ortega, Michelle Guachamin, and Jimmy Velasteguí. HMOA 2165, HMOA

2166 (Fig 1C) and HMOA 2168 were collected at RBCC, 0.93775°S, 77.94891°W, 2193 m altitude, on 16 Nov 2018 by Salomón Ramírez, Grace C. Reyes-Ortega and María José Sánchez.

Diagnosis. *Atopophrynus ikiam* sp. nov. is diagnosed by the following characters: (1) Shagreen skin on dorsum, lacking tubercles; skin of belly weakly areolate; discoidal fold not evident; dorsolateral folds present; (2) tympanic membrane and annulus absent; (3) snout moderately long, subacuminate in dorsal view, truncated in profile; *canthus rostralis* angular in dorsal and lateral view; (4) upper eyelid with low, non-conical tubercles, slightly narrower than inter-orbital distance; cranial crests absent; (5) dentigerous processes of vomers absent; (6) vocal slits, vocal sac and nuptial pads absent; (7) short, stocky fingers; first finger shorter than the second one; terminal discs slightly expanded (absent on Finger I); lateral fringes present; terminal phalanges T-shaped; supernumerary tubercles absent; (8) fingers bearing broad lateral fringes; webbing present; (9) ulnar tubercles absent; (10) heel and tarsus with non-conical tubercles; lacking folds; (11) two smaller metatarsal tubercles present, inner elliptical, about 1.5 times the outer tubercle; supernumerary plantar tubercles absent; (12) toes with lateral fringes; basal webbing evident between Toes II-V; discs slightly smaller than those on fingers; Toe III and V about equal in length; Toe I weak, concealed externally and adherent to Toe II; (13) in life, the dorsum is reddish brown with green and red matte markings (spotted, chevrons on back and diagonal bars on the hindlimbs); pale dorsolateral folds; groin and anterior surfaces of thighs uniformly brownish with red spots; ventral surfaces pale reddish brown; coppery iris rounded by golden ring. In preservation, pale cream

dorsum spotted of dark brown; posterior surfaces of thighs similar to dorsum and posterior surfaces cream, slightly spotted with dark brown; venter uniformly cream; (14) SVL 10.82 mm from holotype (Fig 3).

Description of holotype. Narrow head, less wide than body; slightly longer than wide; snout moderately long, subacuminate in dorsal view, truncated in lateral view; distance from posterior margin of nostril to anterior margin of eye more than double of eye diameter; *canthus rostralis* evident, angular in dorsal and lateral view; lips not enlarged; nostrils directed anterolaterally, protuberant; upper eyelid lacking tubercles, width of upper eyelid 66.2% of interorbital distance; interorbital area lacking dermal folds, IOD 43.3% of head width; large eye, its diameter is about 48.8% of head length; no interocular fold; cranial crests absent. Tympanic membrane and annulus absent; dentigerous processes of vomers absent; vocal slits, vocal sac and nuptial pads absent. Skin on dorsum shagreen, lacking tubercles but bearing dorsolateral folds; skin of belly weakly areolate; discoidal fold not evident; no thoracic fold; skin on flanks shagreen; ventral surfaces of belly, chest and throat, weakly areolate; skin on ventral surfaces of thighs weakly areolate. Forearm slender; fingers short and stocky; terminal discs expanded, more wide than long, evident on Fingers III-IV, distinctive smaller on Finger II than those on other fingers and absent on Finger I; fingers with well-developed lateral fringes; relative length of Fingers I<II<IV<III; subarticular tubercles not evident; supernumerary tubercles absent; palmar and thenar tubercle smaller and rounded; outer edge of forearms weakly areolate with non-conical tubercles; knee and heel with non-conical tubercles; outer and inner edge of tarsus, smooth. Hind limbs

relatively slender; tibia length in about 45.7% of SVL; foot length in about 40.9% of SVL; no outer tarsal tubercles; inner tarsal fold absent; two smaller metatarsal tubercles present, about 1.5 times the outer tubercle; supernumerary tubercles absent; subarticular tubercles scarcely distinguishable; toes with lateral fringes; evident webbing between Toes IV-V; rudimentary, basal webbing between Toes II-IV; webbing absent between Toes I-II; pads of Toes III-V more wide than long, discs slightly smaller than those on fingers; relative lengths I<II<III<V<IV; Toe I weak, concealed externally and adherent to Toe II.

Measurements (in mm) of holotype. Specimen HMOA 2040 is an adult female with the following measurements: SVL = 10.82; HW = 3.56; HL = 3.4; ED = 1.66; IOD = 1.54; IND = 1.52; EN= 0.6; SL = 1.56; UEW = 1.02; NS = 0.96; HAL = 2.52; THL = 5.6; TL = 4.94; FL = 4.42; Toe4DW = 0.44. Proportions: HL/SVL= 0.31; HW/HL= 1.04; THL/SVL= 0.52; TL/SVL= 0.46; FL/SVL= 0.41; EN/HL= 0.18; ED/HL= 0.49; IOD/HW= 0.43.

Coloration in life. The dorsum is reddish brown with two red matte chevrons on back and green matte spots, opaque green diagonal bars on the hindlimbs; three reddish spots in head; pale dorsolateral folds delineated with pale red as a continuous line from face to groin; two diagonal strips in the flanks, one from eye to axilla and the other from eye around arm to medium flank; reddish canthal stripe; yellowish cream arms without stripes; ventral surfaces pale reddish brown with reddish freckles, cream spots with red points toward the flanks and similar spots towards the dump; yellowish ventral surfaces of legs and arms with reddish freckles; black points on the palms and white dermal shield; coppery iris rounded by golden ring.

Coloration in preservation. Dorsum dark brown with smaller white spaces; flanks brownish cream with scattered cream spaces; dark brown cloacal region in dorsal view; dorsal chevrons absent. Dark brown sides of head like the dorsum, slightly spotted on the snout; black upper eyelid; anterior surfaces of thighs like flanks with scattered cream spaces, and the superior surfaces uniform cream with scarce tiny light brown points, groin cream. Thenar, palmar and the two metatarsal tubercles marked with a black point. Venter, chest, throat, ventral surfaces of arms and palms uniform cream; posterior surfaces of tarsus and plantar surfaces scattered spotted of light brown.

Variation. Measurements and proportions of reviewed specimens are shown in Table 3. Clearly, individuals of the new species are smaller, 10.64-10.98 (average = 10.84 mm, n = 4) mm in SVL, than type species of *Atopophrynus syntomops*, 19.52 mm in SVL. Additionally, the posterior foot membranes of the new species are more reduced than *A. syntomops* (Fig 4).

Etymology. The specific name, "*ikiam"*, assigned to the new species means "forest" in Shuar, one of the indigenous languages spoken in the Ecuadorian Amazonia. This word alludes to the habitat where this species lives, located in the pristine and largely unexplored Colonso-Chalupas Biological Reserve, next to the campus of the Universidad Regional Amazónica Ikiam, in the Amazonian slopes of the northeastern Andes.

Natural history and distribution. *Atopophrynus ikiam* sp. nov. is known from a single locality in the Amazonian montane evergreen forest of the northeastern Andes of Ecuador in Napo [70], at approximately 2200 m altitude (Fig 2). This ecosystem is also known as cloud forest due to the cloud covers and haze [70], where several families of epiphytes from the families

Orchidaceae, Bromeliaceae, and Araceae are diverse [71]. According to field notes, all collected specimens of the new species were found active at night on bromeliad leaves (Bromeliaceae) found at about 1.5 m above the ground (Fig 2B). The holotype HMOA 2040 was collected on the night of Dec 20th, 2016 while it was attending a clutch of eggs. The symmetrical transparent egg jelly contained eight eggs of cream lemon color. The HMOA 2055 specimen was collected in a bromeliad leaf on the Dec 21th, 2016. Two years later, three individuals corresponding to HMOA 2165, HMOA 2166 and HMOA 2168 were collected on the night of Nov 16th, 2018 on bromeliad leaves (Bromeliaceae). Since the first two individuals were found attending a clutch of eggs, we hypothesize that their reproductive period occurs during November and December. In the two fieldtrips, at least one female was found attending a clutch of eggs (Fig 1). The first clutch of eggs registered in 2016 was attended by the female HMOA 2040, while in 2018 the male HMOA 2065 and the female HMOA 2066 were together attending the same clutch of eggs. Thus, I suggest that parental care in *Atopophrynus ikiam* sp. nov. is performed by females, and possibly sometimes the male may also be involved. However, we need more observations and data to confirm this assumption. Based on the single known locality of distribution and the threats on nearby areas due by deforestation in the surrounding buffer on the Colonso-Chalupas Biological Reserve, it is proposed to consider this new species as Vulnerable by criteria D2 [72], for "restricted number of locations with a plausible future threat that could drive the taxon to Critically Endangered (CR) in a very short time".

Phylogenetic analyses and divergence time

In order to determine the phylogenetic relationships of *Atopophrynus ikiam* sp. nov., an evolutionary tree was reconstructed based on molecular data from nuclear and mitochondrial DNA. The dataset consisted of 122 taxa and 1703 base pairs, with 566 bp corresponding to gene 12S, 563 bp to 16S, and 574 bp to RAG-1. The BI and ML analyses resolved the phylogenetic position of *Atopophrynus ikiam* sp. nov. placing it within the *Crossodactylus* + *Atopophrynus* group (Fig 5). This group recovered a high bayesian posterior probability (0.96 node value in Fig 5), while the ML bootstrap value has a low support (<50). Relationships of this clade with other families remain unresolved as I recovered a polytomy in all internal groups (Fig 5). The phylogenetic reconstruction only suggests *Crossodactylus* as the sister clade of *Atopophrynus ikiam* sp. nov. (Fig 5). This study suggests Hylodidae (*Hylodes* + *Megaelosia* + *Crossodactylus*) as a paraphyletic group. The molecular phylogeny recovers two paraphyletic groups in two clades: *Hylodes* + *Megaelosia* (PP=1; Bootstrap= 0.81), and *Crossodactylus* + *Atopophrynus ikiam* sp. nov, (PP=0.96; Bootstrap <50). Within these clades, neither *Hylodes*, *Megaelosia* nor *Crossodactylus* formed monophyletic groups. *Megaelosia apuana*, *M. jordanensis* and *M. boticariana* conform a clade strongly supported by posterior probabilities (S1 Fig), but the *M. goeldii* was inserted into the *Hylodes* group.

Despite weak support between major family groups, the relationships of closely related clades were congruent (Fig 5). The analysis grouped the Terrarana clade with three other families: Craugastoridae (*Lynchius* + *Oreobates* + *Bryophryne* + *Phrynopus* + *Pristimantis* + *Microkayla*), Brachycephalidae (*Brachycephalus* + *Ischnocnema*) and

Eleutherodactylidae (*Diasporus* + *Eleutherodactylus* + *Adelophryne*). The bayesian posterior probabilities ≥ 0.95 support the Terrarana clade and its relationships of each family. The closest relative of Terrarana was reconstructed with low support as Hemiphractidae (0.61 ML bootstrap value, Fig 5). The Hemiphractidae (*Cryptobatrachus* + *Hemiphractus* + *Gastrotheca* + *Stefania*) group was recovered with strong bayesian probability support (BI ≥ 0.95, Fig 5), but the relationships among genera were not supported. The Centrolenidae (*Centrolene* + *Nymphargus* + *Cochranella* + *Hyalinobatrachium*) and Allophrynidae (*Allophryne*) groups were recovered as sister taxa with a high bayesian probability, but low ML value (BI ≥ 0.95, ML ≤ 0.95, Fig 5). Ceratophryidae (*Chacophrys* + *Lepidobatrachus* + *Ceratophrys*), Alsodidae (*Eupsophus* + *Alsodes*) and Telmatobiidae (*Telmatobius*) were grouped, but support values were below 0.5 for BI and ML. Also, *Lepidobatrachus*, *Ceratophrys* and *Alsodes* were recovered as paraphyletic groups (Fig 5). Hylidae (*Cruziohyla* + *Phyllomedusa* + *Dryophytes* + *Acris* + *Litoria* + *Dendropsophus* + *Exerodonta* + *Sphaenorhynchus* + *Trachycephalus*) is recovered as monophyletic group, and Leptodactylidae grouped *Pleurodema* + *Engystomops*, but their support values were low (BI ≤ 0.95). Bufonidae (*Incilius* + *Rhinella* + *Anaxyrus* + *Duttaphrynus* + *Melanophryniscus*) is a group strongly supported by BI analysis (BI ≥ 0.95), but closely related groups, like *Rhinella* and *Anaxyrus,* were recovered as paraphyletic. Dendrobatidae and Aromobatidae were linked with considerable support, while Cycloramphidae was not linked with any major group.

The phylogenetic relationships among families reconstructed by the chronogram (Fig 6) are similar to those obtained by the phylogenetic tree (S2 Fig and Fig 5). However, there

are some relationships recovered in Alsodidae, Hylidae and Hylodidae that differs between the chronogram (Fig 6) and the phylogenetic tree (Fig 5). In the chronogram for Alsodidae, *Alsodes* and *Eupsophus* are each composed of monophyletic taxa (Fig 6), in contrast to the phylogenetic tree, where the monophyly is only recovered for *Eupsophus* (Fig 5). The relationships recovered for the Hylidae group also differs between the chronogram and phylogenetic tree. In the chronogram, *Dendropsophus berthalutzae* + *Exerodonta juanitae* are recovered as the sister group of *Sphaenorhynchus pauloalvini* + *S. platycephalus*, *Acris crepitans* + *Dryophytes arenicolor* as the sister group of *Trachycephalus venulosus*, and lastly, *Cruziohyla calcarifer* + *Phyllomedusa hypochondrialis* as the sister group of *Litoria caerulea*. Additionally, the phylogenetic tree and chronogram reconstructions recover Hylodidae as paraphyletic group, with some exceptions in the topology between analyses. In the chronogram, the group *Hylodes* + *Megaelosia* has a different arrangement within the Hylodidae group. The *Hylodes amnicola* + *H. perere* relationship is recovered as a sister clade to *H. japi*, *H. ornatus* and *H. sazimai*. This arrangement was reconstructed like a polytomy in the phylogenetic tree (S1 Fig). Another difference between the chronogram and the phylogeny is that in the chronogram *H. asper* + *H. nasus* and *H. caete* + *H. charadranaetes* are sister clades, and these form a sister group with *H. phyllodes*. For the last clade of Hylodidae, *H. pipilans* + *H. uai* is recovered as the sister clade of *Megaelosia goeldii*, and *H. meridionalis* + *H. perplicatus*. A consistent pattern between the chronogram and phylogenetic tree was the monophyly of *Hylodes* + *Megaelosia* (BI ≥ 0.95). Regarding the new species, *Atopophyrnus* was recovered as sister taxon of *Crossodactylus* in the phylogenetic tree and

the chronogram (Fig 5 and 6). However, *Crossodactylus* is recovered as a monophyletic group sister to *Atopophrynus ikiam* sp. nov., in contrast to the phylogenetic tree, where *Crossodactylus* is a paraphyletic group.

The chronogram reveals that *Atopophrynus ikiam* sp. nov. diversified during the Eocene-Oligocene transition around 33.48 Myr (Fig 6), with its closest related taxa being *Crossodactylus* and *Thoropa*. The divergence time between *Thoropa* and *Crossodactylus* + *Atopophrynus ikiam* sp. nov. dates back to 46.87 Mya while the ancestor of the *Hylodes* and *Megaelosia* group dates back to the Eocene around 36.94 Mya. Thus, the analyses suggest that *Atopophrynus* could be related to ancient lineages of tropical anurans. The relationships recovered for the ingroup in the chronogram topology are supported by a high Bayesian posterior probability of 0.93. In general, these exceptions in the topology of the chronogram that are not consistent with the phylogenetic tree may reflect the uncertainty in the BI and ML support values for close evolutionary relationships.

Ancestral area reconstruction

In order to determine the dispersal patterns of *Atopophrynus ikiam* sp. nov. and its related groups, I reconstructed the ancestral biogeographical areas using a statistical dispersal-vicariance analysis (S-DIVA) [67]. The ancestral area reconstruction shows the biogeographical relationships according to the regionalization of areas for South America in the Neotropical region (Fig 7). The S-DIVA analysis inferred that the ancestral area for the *Crossodactylus* + *Atopophrynus* lineage is located in the Brazilian Shield (Fig 7). Patterns of

historical biogeography reveal a first dispersal event from the Brazilian Shield during the Eocene-Oligocene transition (*Thoropa* → *Crossodactylus + Atopophrynus*, ca. 46.8 Mya), followed by a vicariance event between the Brazilian Shield and northeastern Andes (*Crossodactylus* |*Atopophrynus,* ca. 33.48 Mya) separated by the Amazon Basin (Fig 7). The topology reveals 69 dispersal and 37 vicariance events, with only one extinction event (Fig 6 and 7).

In general, the Hylidae, Bufonidae, Leptodactylidae, Telmatobiidae, Hemiphractidae and Terrarana groups comprise species with diverse ancestral geographical areas and wide distributions (Fig 7). This is in contrast to the Alsodidae and Ceratophryidae lineages, which show the Austral Region as their ancestral area, from where dispersal took place to (H) Central Occidental Andes, (K) Brazilian Shield, (G) Central Oriental Andes, (B) Guiana Shield and (C) Amazon Basin (Fig 7).

Discussion

Herein, I describe a new species of *Atopophrynus* from the Amazonian slopes of Ecuador based on morphological and molecular evidence. The new species, *A. ikiam* sp. nov., is associated to the genus *Atopophrynus* based in a reduction of the first toe regarding to the type series of *A. syntomops,* from Colombia. The "concealment" of the first toe in *A. syntomops* has been linked with another *incertae sedis* lineage, *Geobatrachus walkeri*, which also has toe I externally fused with toe II [44]. However, this character status has been

questionable [38] because *A. syntomops* presents a miniaturization of the metatarsal and a reduction in the size of the phalanges, having a complete phalangeal formula: 2-2-3-4-3 [43]. In contrast, *G. walkeri* presents a shortening in the metatarsal and penultimate phalanx, and a loss of the distal phalanx, having the phalangeal formula: 1-2-3-4-3 [38]. Thus, I suggest that *A. ikiam* sp. nov. is more related to *A. syntomops* than *G. walkeri*, due to the miniaturization of the metatarsal and a reduction in the size of the phalanges observed (Fig 3 and 4). However, *Atopophrynus ikiam* has a reduced foot membrane and a smaller body size (SVL) compared to *A. syntomops*.

From a biogeographical point of view, the distribution of *A. ikiam* sp. nov. seems to be more related to the habitat of the type locality of *A. syntomops*, than *G. walkeri*. *Geobatrachus walkeri* was described from the Santa Marta mountains [73], an isolated mountain range separated from the Andes towards to the Caribbean coast at northern Colombia. While *A. syntomops* was described from the top, 2780 m altitude, of the Cordillera Central in Colombia [42], I suspect it would be more related to the habitat of *A. ikiam* sp. nov. During fieldwork, I sampled extensively along an altitudinal gradient, where no individuals of the new species were found below 2000 m altitude. I have additionally searched in other areas in Ecuador (outside my study area) with similar habitat characteristics above 2000 m altitude, including on several bromeliads morphotypes of the family Bromeliaceae during the reproductive season, but never found additional individuals. Thus, my observations suggest a distribution restricted to the montane evergreen forest in the Guacamayos range located on the Amazonian slopes of the northeastern Andes, above 2000 m altitude in Ecuador.

Amphibian species with limited geographical distributions tend to be more susceptible to disappear in climate change scenarios [2,74–78], as could be the case of *Atopophrynus ikiam* sp. nov. In several global analyses, amphibians with limited distributions are the most susceptible to extinction [2,75–78], but the species of small size, limited mobility, and restricted local distributions seem to be the most vulnerable to the loss of geographical range [74], and therefore to a higher risk of extinction. It is known that extinction risk could be higher for amphibians with poor data [78], as is the case of *A. ikiam* sp. nov., therefore suggesting a conservation category as Vulnerable based on restriction on its distribution and threats on nearby areas in the Reserva Biológica Colonso Chalupas.

The evolutionary relationships of the family Hylodidae have been discussed in previous studies based on morphological [79–81] and molecular evidence [39,40,82–84]. Earlier studies proposed *Crossodactylus* as *Hylodes* sister taxon [79–81]. However, more recent phylogenetic analyses suggest that *Megaelosia* is more closely related to *Hylodes*, and that *Crossodactylus* is an external taxa [40,82–84]. While Hylodidae (*Hylodes* + *Megaelosia* + *Crossodactylus*) has been previously considered as a monophyletic group [40,82], this study did not support this hypothesis. In the two clades recovered by the molecular phylogeny, *Hylodes* + *Megaelosia*, and *Crossodactylus* + *Atopophrynus ikiam* sp. nov., neither *Hylodes*, *Megaelosia* nor *Crossodactylus* formed monophyletic groups as seen in previous studies [40,82]. Also, the analyses recovered the insertion of *Megaelosia goeldii* into the *Hylodes* group suggesting that these genera are not monophyletic, as suggested in a previous study [40]. In this study, based on genetic evidence, *Atopophrynus ikiam* sp. nov., is tentatively

assigned to the Hylodidae family by its close relation with species of *Crossodactylus*. Members of the genus *Crossodactylus* were recovered as paraphyletic in the ML phylogeny, being *C. werneri* the closest relative of *Atopophrynus ikiam* sp. nov., followed by *C. caramaschii* + *C. aeneus* + *C. schmidti*. However, in the chronogram, members of *Crossodactylus* are recovered as monophyletic, whereas *Atopophrynus ikiam* sp. nov. is recovered as their sister taxa together with members of the genus *Thoropa* (Fig 6). The topology of the chronogram shows a long branch for *Atopophrynus*, which can be attributed to an information gap in the analysis. To minimize long branch attraction, it is possible to increase the density of sampling in the group by adding additional taxa, so the longest branch will be subdivided [85]. In a general sense, the reconstruction of major family groups is consistent with previous studies [39,40].

Interestingly, the new species is part of one of the most ancient clades within Anura (Hyloidea lineage) [14,40], with a South American origin in Paleogene around 33.48 Mya. On the other hand, all the members of *Crossodactylus* are distributed along the Southeastern Atlantic forest in Brazil with a divergence time of 20.74 Mya. The divergence time between *Thoropa* and *Crossodactylus* + *Atopophrynus ikiam* sp. nov. dates back to 46.87 Mya, and between *Hylodes* and *Megaelosia* to 36.94 Mya, both corresponding to the Eocene. *Hylodes* and *Megaelosia* are more ancestral to *Thoropa* and *Crossodactylus* + *Atopophrynus* group. These patterns are in contrast to other studies showing several Anuran groups having diversified in the Neogene as a consequence of geological events in the Andean region [30–35]. The diversification time of the new species corresponds to two episodes of dispersal and

further vicariance occurred along the Amazon basin, congruent with major geological events. First, a dispersion event occurring from eastern South America through the pan-Amazonia extension in the Paleocene-Eocene transition, ca. 46.8 Mya (*Thoropa* → *Crossodactylus + Atopophrynus*). This historical event reveals a connection that may have allow ancestors of *Atopophrynus* reach the current Ecuadorian region, even before the northern uplift of Andes [86]. Also, it could be that the species of Hylodidae diversified in the eastern Amazonia during this episode, a hypothesis congruent with the divergence time of *Hylodes*, *Megaelosia* and *Crossodactylus* dating back to the Eocene. Second, a vicariance event in the Oligocene ca. 33.48 Mya (*Crossodactylus* |*Atopophrynus)* where populations from the western Andes were isolated from the eastern Amazonian Shield, due to the initial uplift of the northern Andes and the origin of Sub Andean river system [7]. In this episode, the populations may have adapted and colonized new habitats created by the changes in the Andean orogeny, which could explain the presence of the *Atopophrynus* lineage in the Colombian and Ecuadorian Andes. Later on, the species from the Atlantic forest and the Andes were isolated by barriers as the uplift Andes and wetlands in the northwestern Amazonia during the Miocene [7,20,21]. Thus, *Atopophrynus* was separated and isolated from *Crossodactylus* lineages in the Amazon Basin (Fig 7), with restricted distributions towards the Amazonian slopes of Ecuador and Colombia (*Atopophrynus*), and the Brazilian Shield (*Crossodactylus*).

Conclusions

The results suggest the major geological events in the Paleogene as the most important events of origin and diversification of the new species (*Atopophrynus ikiam* sp. nov.) related to ancient South American clades within Anura. I identified two events: a dispersal event from eastern South America through the pan-Amazonia in the Paleocene-Eocene and a vicariance event from the western Andes and eastern Amazonian Shield due to the initial uplift of the northern Andes and the origin of Sub Andean river system in the Oligocene. Therefore, I identify the important role of paleogeological changes in South America as promoters of the speciation for *Atopophrynus*. It is important, however, to incorporate more information to understand with more certainty the taxonomy and systematics of the group. In a conservation approach, it is necessary to join efforts to preserve the new species restricted to the montane evergreen forest in the Guacamayos range, surrounding the Amazonian slopes of the northeastern Andes in Ecuador. The information provided in this study opens the way to future work focusing on additional biogeographic analyses, systematics and natural history studies on an unexplored region in Ecuador.

References

1. Jenkins CN, Pimm SL, Joppa LN. Global patterns of terrestrial vertebrate diversity and conservation. PNAS. 2013;110: E2602–E2610.

2. Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, Joppa LN, et al. The biodiversity of species and their rates of extinction, distribution, and protection. Science (80). 2014;344: 1246752.

3. Antonelli A, Zizka A, Carvalho FA, Scharn R, Bacon CD, Silvestro D, et al. Amazonia is the primary source of Neotropical biodiversity. Proc Natl Acad Sci U S A. 2018;115: 6034–6039.

4. van der Hoek Y, Gaona G V., Martin K. The diversity, distribution and conservation status of the tree-cavity-nesting birds of the world. Divers Distrib. 2017;23: 1120–1131.

5. Kreft H, Jetz W. Global patterns and determinants of vascular plant diversity. Proc Natl Acad Sci U S A. 2007;104: 5925–5930.

6. Antonelli A, Sanmartín I. Why are there so many plant species in the Neotropics? Taxon. 2011;60: 403–414.

7. Hoorn C, Wesselingh FP, ter Steege H, Bermudez MA, Mora A, Sevink J, et al. Amazonia Through Time: Andean Uplift, Climate Change, Landscape Evolution, and Biodiversity. Science (80). 2010;330: 927–931.

8. Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Kent J. Biodiversity hotspots for conservation priorities. Nature. 2000;403: 853–858.

9. Widdowson M, Cox KG. Uplift and erosional history of the Deccan Traps, India: Evidence from laterites and drainage patterns of the Western Ghats and Konkan Coast. Earth Planet Sci Lett. 1996;137: 57–69.

10. Prasad V, Farooqui A, Tripathi SKM, Garg R, Thakur B. Evidence of late Palaeocene-early eocene equatorial rain forest refugia in southern Western Ghats, India. J Biosci. 2009;34: 777–797.

11. Rahbek C, Borregaard MK, Antonelli A, Colwell RK, Holt BG, Nogues-Bravo D, et al. Building mountain biodiversity: Geological and evolutionary processes. Science (80-). 2019;365: 1114–1119.

12. Fjeldså J, Bowie RCK, Rahbek C. The Role of Mountain Ranges in the Diversification of Birds. Annu Rev Ecol Evol Syst. 2012;43: 249–265.

13. Pyron RA. Biogeographic analysis reveals ancient continental vicariance and recent oceanic dispersal in amphibians. Syst Biol. 2014;63: 779–797.

14. Feng YJ, Blackburn DC, Liang D, Hillis DM, Wake DB, Cannatella DC, et al. Phylogenomics reveals rapid, simultaneous diversification of three major clades of Gondwanan frogs at the Cretaceous–Paleogene boundary. Proc Natl Acad Sci U S A. 2017;114: E5864–E5870.

15. Van Bocxlaer I, Biju S, Loader SP, Bossuyt F. Toad radiation reveals into-India dispersal as a source of endemism in the Western Ghats-Sri Lanka biodiversity hotspot. BMC Evol Biol. 2009;9: 131.

16. Bossuyt F, Meegaskumbura M, Beenaerts N, Gower DJ, Pethiyagoda R, Roelants K, et al. Local endemism within the Western Ghats-Sri Lanka biodiversity hotspot. Science (80). 2004;306: 479–481.

17. Pérez-Escobar OA, Chomicki G, Condamine FL, Karremans AP, Bogarín D, Matzke NJ, et

al. Recent origin and rapid speciation of Neotropical orchids in the world's richest plant biodiversity hotspot. New Phytol. 2017;215: 891–905.

18. Badgley C. Tectonics, topography, and mammalian diversity. Ecography (Cop). 2010;33: 220–231.

19. Elmer KR, Bonett RM, Wake DB, Lougheed SC. Early Miocene origin and cryptic diversification of South American salamanders. BMC Evol Biol. 2013;13: 59.

20. Jaramillo C, Romero I, D'Apolito C, Bayona G, Duarte E, Louwye S, et al. Miocene flooding events of western Amazonia. Sci Adv. 2017;3: 1–12.

21. Latrubesse EM, Cozzuol M, da Silva-Caminha SAF, Rigsby CA, Absy ML, Jaramillo C. The Late Miocene paleogeography of the Amazon Basin and the evolution of the Amazon River system. Earth-Science Rev. 2010;99: 99–124.

22. Chaves JA, Weir JT, Smith TB. Diversification in Adelomyia hummingbirds follows Andean uplift. Mol Ecol. 2011;20: 4564–4576.

23. Ferreira M, Aleixo A, Ribas CC, Santos MPD. Biogeography of the Neotropical genus Malacoptila (Aves: Bucconidae): the influence of the Andean orogeny, Amazonian drainage evolution and palaeoclimate. J Biogeogr. 2017;44: 748–759.

24. De-Silva DL, Elias M, Willmott K, Mallet J, Day JJ. Diversification of clearwing butterflies with the rise of the Andes. J Biogeogr. 2016;43: 44–58.

25. Chazot N, Willmott KR, Lamas G, Freitas AVL, Piron-Prunier F, Arias CF, et al. Renewed diversification following Miocene landscape turnover in a Neotropical butterfly radiation. Glob Ecol Biogeogr. 2019;28: 1118–1132.

26. Salgado-Roa FC, Pardo-Diaz C, Lasso E, Arias CF, Solferini VN, Salazar C. Gene flow and Andean uplift shape the diversification of Gasteracantha cancriformis (Araneae: Araneidae) in Northern South America. Ecol Evol. 2018;8: 7131–7142.

27. Antonelli A, Nylander JAA, Persson C, Sanmartín I. Tracing the impact of the Andean uplift on Neotropical plant evolution. Proc Natl Acad Sci U S A. 2009;106: 9749–54. Available: http://www.ncbi.nlm.nih.gov/pubmed/19470489

28. Sanín MJ, Kissling WD, Bacon CD, Borchsenius F, Galeano G, Svenning JC, et al. The Neogene rise of the tropical Andes facilitated diversification of wax palms (Ceroxylon: Arecaceae) through geographical colonization and climatic niche separation. Bot J Linn Soc. 2016;182: 303–317.

29. Hutter CR, Lambert SM, Wiens JJ. Rapid diversification and time explain amphibian richness at different scales in the Tropical Andes, Earth's most biodiverse hotspot. Am Nat. 2017;190: 828–843.

30. Santos J, Coloma LA, Summers K, Caldwell JP, Ree R, Cannatella DC. Amazonian amphibian diversity is primarily derived from late Miocene Andean lineages. PLoS Biol. 2009;7: e1000056.

31. Castroviejo-Fisher S, Guayasamin JM, Gonzalez-Voyer A, Vilà C. Neotropical diversification seen through glassfrogs. J Biogeogr. 2014;41: 66–80.

32. Vallinoto M, Sequeira F, Sodré D, Bernardi JAR, Sampaio I, Schneider H. Phylogeny and biogeography of the Rhinella marina species complex (Amphibia, Bufonidae) revisited: implications for Neotropical diversification hypotheses. Zool Scr. 2010;39: 128–140.

33. Maciel NM, Collevatti RG, Colli GR, Schwartz EF. Late Miocene diversification and phylogenetic relationships of the huge toads in the Rhinella marina (Linnaeus, 1758) species group (Anura: Bufonidae). Mol Phylogenet Evol. 2010;57: 787–797.

34. Fouquet A, Loebmann D, Castroviejo-Fisher S, Padial JM, Orrico VGD, Lyra ML, et al. From Amazonia to the Atlantic forest: Molecular phylogeny of Phyzelaphryninae frogs reveals unexpected diversity and a striking biogeographic pattern emphasizing conservation challenges. Mol Phylogenet Evol. 2012;65: 547–561.

35. Fouquet A, Santana Cassini C, Fernando Baptista Haddad C, Pech N, Trefaut Rodrigues M. Species delimitation, patterns of diversification and historical biogeography of the Neotropical frog genus Adenomera (Anura, Leptodactylidae). J Biogeogr. 2014;41: 855–870.

36. Crawford AJ, Smith EN. Cenozoic biogeography and evolution in direct-developing frogs of Central America (Leptodactylidae: Eleutherodactylus) as inferred from a phylogenetic analysis of nuclear and mitochondrial genes. Mol Phylogenet Evol. 2005;35: 536–555.

37. Mendoza ÁM, Ospina OE, Cárdenas-Henao H, García-R JC. A likelihood inference of historical biogeography in the world's most diverse terrestrial vertebrate genus: Diversification of direct-developing frogs (Craugastoridae: Pristimantis) across the Neotropics. Mol Phylogenet Evol. 2015;85: 50–58.

38. Padial JM, Grant T, Frost DR. Molecular systematics of terraranas (Anura: Brachycephaloidea) with an assessment of the effects of alignment and optimality

criteria. 2014.

39. Heinicke MP, Duellman WE, Trueb L, Means DB, Macculloch RD, Hedges SB. A new frog family (Anura: Terrarana) from South America and an expanded direct-developing clade revealed by molecular phylogeny. Zootaxa. 2009;2211: 1–35.

40. Pyron AR, Wiens JJ. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol Phylogenet Evol. 2011;61: 543–583.

41. Heinicke MP, Duellman WE, Hedges SB. Major Caribbean and Central American frog faunas originated by ancient oceanic dispersal. Proc Natl Acad Sci U S A. 2007;104: 10092–10097.

42. Lynch JD, Ruíz-Carranza PM. A new genus and species of poison-dart frog (Amphibia: Dendrobatidae) from the andes of northern Colombia. Proc Biol Soc Washington. 1982;95: 557–562.

43. Myers CW, Ford LS. On Atopophrynus, a recently described frog wrongly assigned to the Dendrobatidae. Am Museum Novit. 1986;2843. Available: http://hdl.handle.net/2246/3578

44. Hedges SB, Duellman WE, Heinicke MP. New World direct-developing frogs (Anura: Terrarana): Molecular phylogeny, classification, biogeography, and conservation. Zootaxa. 2008.

45. Wiens JJ, Donoghue MJ. Historical biogeography, ecology and species richness. Trends Ecol Evol. 2004;19: 639–644.

46. Santos CMD, Amorim DS. Why biogeographical hypotheses need a well supported phylogenetic framework: a conceptual evaluation. 2007;47: 63–73.

47. Sanmartín I. Historical Biogeography: Evolution in Time and Space. Evol Educ Outreach. 2012;5: 555–568.

48. Duellman WE, Lehr E. Terrestrial Breeding Frogs (Strabomantidae) in Peru. Münster, Alemania: Natur und Tier-Verlag GmbH; 2009.

49. Watters JL, Cummings ST, Flanagan RL, Siler CD. Review of morphometric measurements used in anuran species descriptions and recommendations for a standardized approach. Zootaxa. 2016;4072: 477–495.

50. Promega Corporation. Wizard® Genomic DNA Purification Kit Techinal Manual. Tech Bull. 2019; 1–19. Available: www.promega.com

51. Promega Corporation. Wizard Genomic DNA Purification Kit Quick Protocol. 2010; 1123–1126.

52. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science (80-). 1988;239: 487–491.

53. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28: 1647–1649.

54. Biomatters Ltd. Geneious Prime 2020.0.5. 2020. Available: www.geneious.com

55. Sayers EW, Beck J, Brister JR, Bolton EE, Canese K, Comeau DC, et al. Database

resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2020;48: D9–D16.

56. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215: 403–410.

57. Maddison WP, Maddison DR. Mesquite: a modular system for evolutionary analysis Version 3.2. 2017. Available: www.mesquiteproject.org

58. Lanfear R, Calcott B, Ho SYW, Guindon S. PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol. 2012;29: 1695–1701.

59. Miller MA, Pfeiffer W, Schwartz T. Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. Gatew Comput Environ Work. 2010.

60. Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, et al. Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. Syst Biol. 2012;61: 539–542.

61. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Syst Biol. 2018;67: 901–904.

62. Zwickl DJ. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Philosophy. 2006; 115.

63. Bouckaert R, Heled J, Kühnert D, Vaughan T, Wu CH, Xie D, et al. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Comput Biol. 2014;10: 1–7.

64. Heath TA. Divergence Time Estimation using BEAST v2 . Dating Species Divergences with the Fossilized Birth-Death Process. BEAST v2 Tutor.

65. Drummond AJ, Rambaut A, Bouckaert RR. Divergence Dating Tutorial with BEAST 2.0. Beast 20. 2013; 19.

66. Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Syst Biol. 2018;67: 901–904.

67. Yu Y, Harris AJ, He X. S-DIVA (Statistical Dispersal-Vicariance Analysis): A tool for inferring biogeographic histories. Mol Phylogenet Evol. 2010;56: 848–850.

68. Yu Y, Harris AJ, Blair C, He X. RASP (Reconstruct Ancestral State in Phylogenies): A tool for historical biogeography. Mol Phylogenet Evol. 2015;87: 46–49.

69. University of California. AmphibiaWeb. In: Berkeley, CA, USA [Internet]. 2020. Available: https://amphibiaweb.org

70. Ministerio del Ambiente del Ecuador. Sistema de Clasificación de Ecosistemas del Ecuador Continental. Quito, Ecuador: Ministerio del Ambiente del Ecuador; 2013.

71. Mogollón H, Guevara J, Remache G. Caracterización vegetal de la Bioreserva del Cóndor. Fund NUMASHIR para la Conserv Ecosistemas Amenazado. 2004; 1–84.

72. IUCN. Guidelines for Using the IUCN Red List Categories and Criteria. In: Version 14. Standards and Petitions Subcommittee of the International Union for the Conservation of Nature. [Internet]. 2019. Available: http://intranet.iucn.org/webfiles/doc/SSC/RedList/RedListGuidelines.pdf

73. Ruthven AG. Description of a new tailless amphibian of the family Dendrobatidae. Occas Pap Museum Zool. 1915;20: 1–3.

74. Zank C, Becker FG, Abadie M, Baldo D, Maneyro R, Borges-Martins M. Climate change and the distribution of neotropical red-bellied toads (Melanophryniscus, Anura, Amphibia): How to prioritize species and populations? PLoS One. 2014;9: 1–11.

75. Wake DB, Vredenburg VT. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci. 2008;105: 11466–11473.

76. Howard SD, Bickford DP. Amphibians over the edge: Silent extinction risk of Data Deficient species. Divers Distrib. 2014;20: 837–846.

77. Ficetola GF, Rondinini C, Bonardi A, Baisero D, Padoa-Schioppa E. Habitat availability for amphibians and extinction threat: A global analysis. Divers Distrib. 2015;21: 302–311.

78. González-del-Pliego P, Freckleton RP, Edwards DP, Koo MS, Scheffers BR, Pyron RA, et al. Phylogenetic and Trait-Based Prediction of Extinction Risk for Data-Deficient Amphibians. Curr Biol. 2019;29: 1557-1563.e3.

79. Ardila-Robayo MC. Status sistematico del genero Geobatrachus Ruthven 1915 (Amphibia: Anura). Caldasia. 1979;12: 383–495.

80. Haas A. Phylogeny of frogs as inferred from primarily larval characters (Amphibia: Anura). Cladistics. 2003;19: 23–89.

81. Nuin PAS, do Val FC. Phylogenetic analysis of the subfamily Hylodinae (Anura, Leptodactylidae) based on morphological characters. Amphib Reptil. 2005;26: 139–

147.

82. Grant T, Frost DR, Caldwell JP, Gagliardo R, Haddad CFB, Kok PJR, et al. Phylogenetic Systematics of Dart-Poison Frogs and Their Relatives (Amphibia: Athesphatanura: Dendrobatidae). Bull Am Museum Nat Hist. 2006;299: 1–262.

83. de Sá FP, Canedo C, Lyra ML, Haddad CFB. A New Species of Hylodes (Anura, Hylodidae) and its Secretive Underwater Breeding Behavior. Herpetologica. 2015;71: 58–71.

84. Malagoli LR, de Sá FP, Canedo C, Haddad CFB. A New Species of Hylodes (Anura, Hylodidae) from Serra do Mar, Southeastern Brazil: The Fourth with Nuptial Thumb Tubercles. Herpetologica. 2017;73: 136–147.

85. Olsen GJ. Parsimony. Encyclopedia of Genetics. Elsevier Science Inc.; 2001. pp. 1415–1419.

86. Coltorti M, Ollier CD. Geomorphic and tectonic evolution of the Ecuadorian Andes. Geomorphology. 2000;32: 1–19.

Tables

Table 1. Primers used in this study listed 5-prime to 3-prime.

Primer	Sequence	Direction
12sH10	CACYTTCCRGTRCRYTTACCRTGTTACGACTT	F
12sL4E	TACACATGCAAGTYTCCGC	R
16sSar - L	CGCCTGTTTATCAAAAACAT	F
16sSbr - H	CCGGTCTGAACTCAGATCACGT	R
RAG1 - R182	GCCATAACTGCTGGAGCATYAT	F
RAG1 - R270	AGYAGATGTTGCCTGGGTCTTC	R

Table 2. Sample details including species, GenBank accession number (new sequences in bold), and phylogenetic reconstruction group.

Species	12s	16s	RAG1	Group
Alytes obstetricans	AY364340	AY364362		Outgroup
Bombina variegata	DQ283249	DQ283249	JF898470	Outgroup
Discoglossus montalentii	JQ626634	AY333714		Outgroup
Uperoleia laevigata		EF107187		Outgroup
Acris crepitans	EF566970	EF566970		Ingroup
Adelophryne gutturosa	EU186679	EU186679		Ingroup
Allophryne ruthveni	AY843564	AY843564		Ingroup
Alsodes barrioi	JX204154	JX204154		Ingroup
Alsodes coppingeri	JX204156	JX204156		Ingroup
Alsodes gargola	JX564852	JX564852*		Ingroup
Alsodes hugoi	JX204169	JX204169		Ingroup
Alsodes igneus	JX204171	JX204171		Ingroup
Alsodes neuquensis	JX204173	JX204173		Ingroup
Alsodes nodosus	JX204174	JX204174		Ingroup
Alsodes pehuenche	JX204177	JX204177		Ingroup
Alsodes tumultuosus	JX204185	JX204185		Ingroup
Alsodes valdiviensis	JX204188	JX204188		Ingroup
Alsodes vanzolinii	JX204189	JX204189		Ingroup
Alsodes verrucosus	JX204191	JX204191		Ingroup
Anaxyrus americanus	DQ158426	DQ158426	KJ609650*	Ingroup

Anaxyrus boreas	DQ158436	DQ158436	KJ609660*	Ingroup
Atopophrynus ikiam sp. nov.	**HMOA2055**	**HMOA2055**	**HMOA2055**	Ingroup
Atopophrynus ikiam sp. nov.	**HMOA2165**	**HMOA2165**		Ingroup
Atopophrynus ikiam sp. nov.		**HMOA2166**	**HMOA2166**	Ingroup
Brachycephalus ephippium	HM216364	HM216365	HM216366	Ingroup
Brachycephalus izecksohni	HQ435683	HQ435696	HQ435725*	Ingroup
Bryophryne bustamantei	MF186295	MF186356	MF186543*	Ingroup
Centrolene venezuelense	EU663359	EU663000		Ingroup
Ceratophrys aurita	KP295606	KP295606*		Ingroup
Ceratophrys cornuta	HQ290947	HQ290947*		Ingroup
Ceratophrys cranwelli	KP295613	KP295613*		Ingroup
Ceratophrys joazeirensis	KP295617	KP295617*		Ingroup
Chacophrys pierottii	KP295624	KP295624*		Ingroup
Cochranella mache	EU663373	EU663013		Ingroup
Crossodactylus aeneus		KM390791		Ingroup
Crossodactylus caramaschii	AY143346	KJ961569*		Ingroup
Crossodactylus schmidti	AY843579	AY843579		Ingroup
Crossodactylus werneri		KU215900		Ingroup
Cruziohyla calcarifer	EF396339*	FJ784495		Ingroup
Cryptobatrachus remotus		KR270416	KR138394*	Ingroup
Dendrobates auratus	AY364565	AY364565		Ingroup
Dendropsophus berthalutzae	AY843607	KM390782*		Ingroup
Diasporus diastema	EU186682	EU186682	EU186752	Ingroup
Dryophytes arenicolor	EF566960	EF566960		Ingroup
Duttaphrynus melanostictus	AY458592	AY458592		Ingroup
Eleutherodactylus cooki	EF493539	EF493539	EF493413*	Ingroup
Eleutherodactylus counouspeus	EF493719	EF493719	EU186760*	Ingroup
Engystomops guayaco	DQ337220*	DQ337220		Ingroup
Engystomops petersi	JN970376*	JN970376		Ingroup
Engystomops puyango	HQ111350*	HQ111350		Ingroup
Engystomops randi	DQ337228*	DQ337228		Ingroup
Eupsophus calcaratus	JX204197	JX204197		Ingroup
Eupsophus contulmoensis	JX204202	JX204202		Ingroup
Eupsophus emiliopugini	JX204204	JX204204		Ingroup
Eupsophus insularis	JX204206	JX204206		Ingroup
Eupsophus migueli	JX204209	JX204209		Ingroup
Eupsophus nahuelbutensis	JX204211	JX204211		Ingroup
Eupsophus roseus	JX204214	JX204214		Ingroup

Eupsophus septentrionalis	JX204218	JX204218		Ingroup
Eupsophus vertebralis	JX204221	JX204221		Ingroup
Exerodonta juanitae		KX423491*		Ingroup
Gastrotheca plumbea	DQ679254	DQ679403*		Ingroup
Hemiphractus helioi	AY843594	AY843594	KR138399*	Ingroup
Hyalinobatrachium fleischmanni	EU663406	EU663045		Ingroup
Hylodes amnicola		KJ961576*		Ingroup
Hylodes asper	KY202787	KU495250		Ingroup
Hylodes caete		KY627902*		Ingroup
Hylodes charadranaetes		KM390793*		Ingroup
Hylodes japi		KJ961571*		Ingroup
Hylodes meridionalis	AY143342	MF624224		Ingroup
Hylodes nasus		KJ961577*		Ingroup
Hylodes ornatus	AY143343	KJ961578		Ingroup
Hylodes perere		KJ961579*		Ingroup
Hylodes perplicatus	AY143341	AY263225		Ingroup
Hylodes phyllodes	KY202790			Ingroup
Hylodes pipilans		KJ961582		Ingroup
Hylodes sazimai	AY143344	KJ961585*		Ingroup
Hylodes uai		KY002953*		Ingroup
Incilius coccifer	DQ158443	DQ158443	KJ609669*	Ingroup
Ischnocnema guentheri	JX267331	JX267497	JX267607*	Ingroup
Ischnocnema hoehnei	JX267345		JX267616	Ingroup
Ischnocnema holti	JX267306	JX267306	JX267617*	Ingroup
Lepidobatrachus asper	KP295626	KP295626*		Ingroup
Lepidobatrachus laevis	DQ283152	DQ283152*		Ingroup
Lepidobatrachus llanensis	KP295641	KP295641*		Ingroup
Litoria caerulea	AY843692	AY843692	EF493446*	Ingroup
Lynchius oblitus	KX470776	KX470783	KX470792*	Ingroup
Mannophryne trinitatis	DQ502131	DQ502131		Ingroup
Megaelosia apuana		KU495387		Ingroup
Megaelosia boticariana		KJ961586*		Ingroup
Megaelosia goeldii	AY143348	KM390796*		Ingroup
Megaelosia jordanensis	MF624238	MF624238		Ingroup
Melanophryniscus rubriventris	KX276233*	KX276312		Ingroup
Melanophryniscus stelzneri	AY325999	AY325999		Ingroup
Microkayla adenopleura	MF186283	MF186340	MF186537*	Ingroup
Nymphargus cochranae	EU663425	EU663061		Ingroup

Oreobates antrum	MH025427	MH025451	MH025436*	Ingroup
Oreobates quixensis	EF493828	EF493662		Ingroup
Phrynopus badius	MG896595	MG896572	MG896619*	Ingroup
Phrynopus bracki	EF493709	EF493709	EF493421*	Ingroup
Phrynopus juninensis	MG896600	MG896577	MG896623*	Ingroup
Phrynopus montium	MG896602	MG896579	MG896625*	Ingroup
Phyllomedusa hypochondrialis	AY843724	AY843724		Ingroup
Pleurodema brachyops	AY843733	AY843733		Ingroup
Pristimantis altamazonicus	MF118673	MF118679	MF118736	Ingroup
Pristimantis brevicrus		MF118674	MF118741	Ingroup
Rhinella arenarum	AY843573	AY843573		Ingroup
Rhinella margaritifera	HM563816	HM563858		Ingroup
Rhinella spinulosa	AY680263	AY680263	KJ609676*	Ingroup
Sphaenorhynchus pauloalvini	MK266750*	MK266750		Ingroup
Sphaenorhynchus platycephalus	MK266746	MK266746*		Ingroup
Stefania evansi	AY843767	KR270433	KR138401*	Ingroup
Stefania scalae	DQ679267	KR270434	KR138402*	Ingroup
Telmatobius chusmisensis		KJ562953*		Ingroup
Telmatobius dankoi	AF145387	KJ562955*		Ingroup
Telmatobius fronteriensis		KJ562959*		Ingroup
Telmatobius marmoratus	AY578822	KJ562964*		Ingroup
Telmatobius vellardi	JX564897	JX564897*		Ingroup
Telmatobius verrucosus	DQ283040	DQ283040		Ingroup
Thoropa miliaris	DQ283331	DQ283331*		Ingroup
Thoropa taophora		MG799615		Ingroup
Trachycephalus venulosus	AY326048	AY326048		Ingroup

*Sequences with the highest percent identity in Blast.

Table 3. Measurements (in mm) and proportions of specimens of Atopophrynus syntomops from Cordillera Central, Colombia, and Atopophrynus ikiam sp. nov. from RBCC, Ecuador. Characters correspond to snout-vent length, SVL; head width, HW; head length, HL; horizontal eye diameter, ED; inter-orbital distance, IOD; internarial distance, IND; eye-nostril distance, EN; snout length, SL; width of upper eyelid, UEW; snout-nostril length, NS; hand length, HAL; thigh length, THL; tibia length, TL; foot length, FL; and toe IV disk width,

Toe4DW. Abbreviation of ICN corresponds to Instituto de Ciencias Naturales of Universidad Nacional de Colombia in Bogotá, and HMOA corresponds to field number.

	A. syntomops	***A. ikiam* sp. nov.**				
Character	ICN 08611	HMOA 2040	*HMOA 2055	HMOA 2165	HMOA 2166	HMOA 2168
	Female	Female	Female	Male	Female	Female
SVL	19.52	10.82		10.98	10.92	10.64
HW	6.84	3.56		4.14	3.82	3.68
HW/SVL	0.35	0.33		0.38	0.35	0.35
HL	6.28	3.4		4.16	3.84	4.02
HL/SVL	0.32	0.31		0.38	0.35	0.38
ED	2.78	1.66		2.1	1.92	1.92
ED/HL	0.44	0.49		0.50	0.50	0.48
IOD	2.28	1.54		1.58	1.54	1.6
IOD/HW	0.33	0.43		0.38	0.40	0.43
IND	2.48	1.52		1.54	1.6	1.6
IND/HW	0.36	0.43		0.37	0.42	0.43
EN	1.32	0.6		0.7	0.6	0.5
EN/HL	0.21	0.18		0.17	0.16	0.12
SL	2.44	1.56		1.88	1.54	1.76
SL/HL	0.39	0.46		0.45	0.40	0.44
UEW	1.58	1.02		1.16	1.02	1.02
UEW/HW	0.23	0.29		0.28	0.27	0.28
NS	1.26	0.96		0.94	0.96	0.92
NS/HL	0.20	0.28		0.23	0.25	0.23
HAL	6.08	2.52	3.5	3.16	3.22	3.2
HAL/SVL	0.31	0.23		0.29	0.29	0.30
THL	8.94	5.6	5.1	5.3	5.14	5.54
THL/SVL	0.46	0.52		0.48	0.47	0.52
TL	8.34	4.94	5.46	5.5	5.34	5.52
TL/SVL	0.43	0.46		0.50	0.49	0.52
FL	7.8	4.42	4.8	4.6	4.28	4.94
FL/SVL	0.40	0.41		0.42	0.39	0.46
Toe4DW	1.08	0.44	0.6	0.58	0.46	0.6
Toe4DW/FL	0.14	0.10	0.13	0.13	0.11	0.12

*Due to a fieldtrip accident related with the container of specimens, the upper part of the body in specimen HMOA 2055 was lost.

Figures

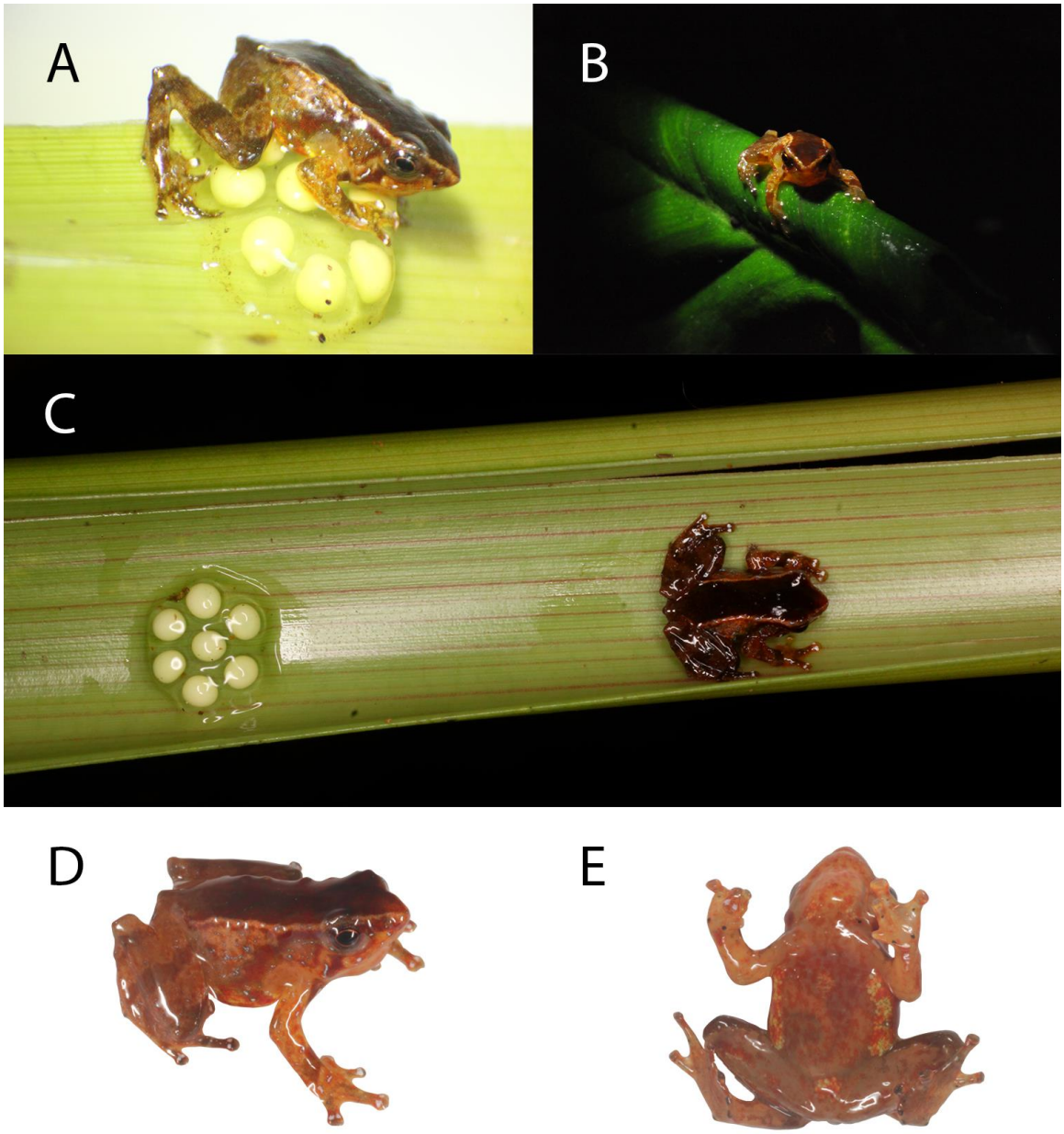


Fig 1. *Atopophrynus ikiam* sp. nov. in life. (A-B) Holotype HMOA 2040 female, taken at night during the fieldtrip in Dec 2016. (C) Paratype HMOA 2166 female photographed at night during the fieldtrip in Nov 2018. (D-E) Holotype HMOA 2040 photographed during the day. Females were observed laying eggs during both fieldtrips.

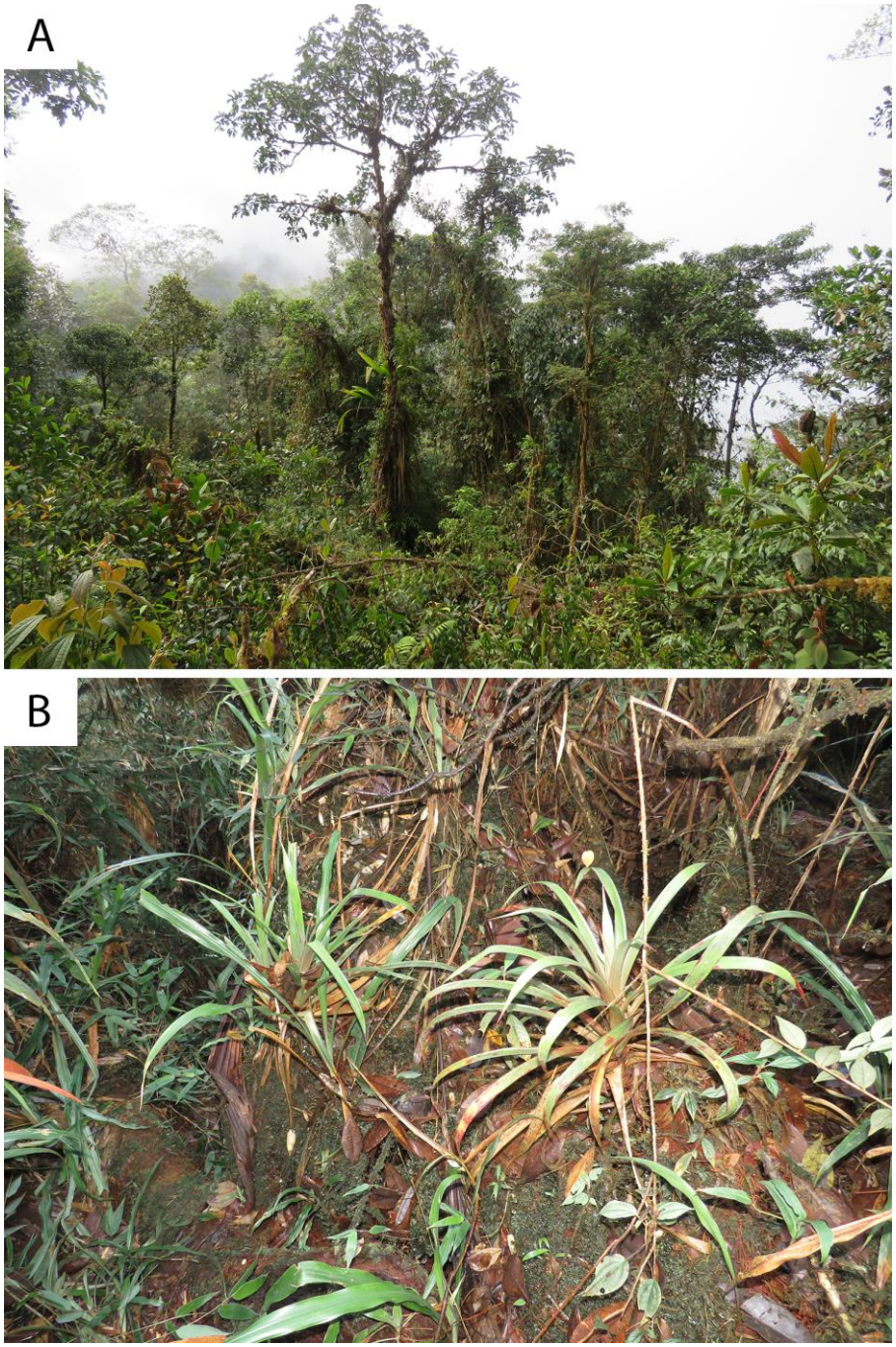


Fig 2. (A) Cloud forest in the Reserva Biológica Colonso Chalupas in eastern Ecuador, the type locality of *Atopophrynus ikiam* sp. nov. (B) Bromeliad species (Bromeliaceae) where the specimens of the new species were found during fieldtrips in 2016 and 2018. In both occasions, the bromeliads were found relatively close to the ground.

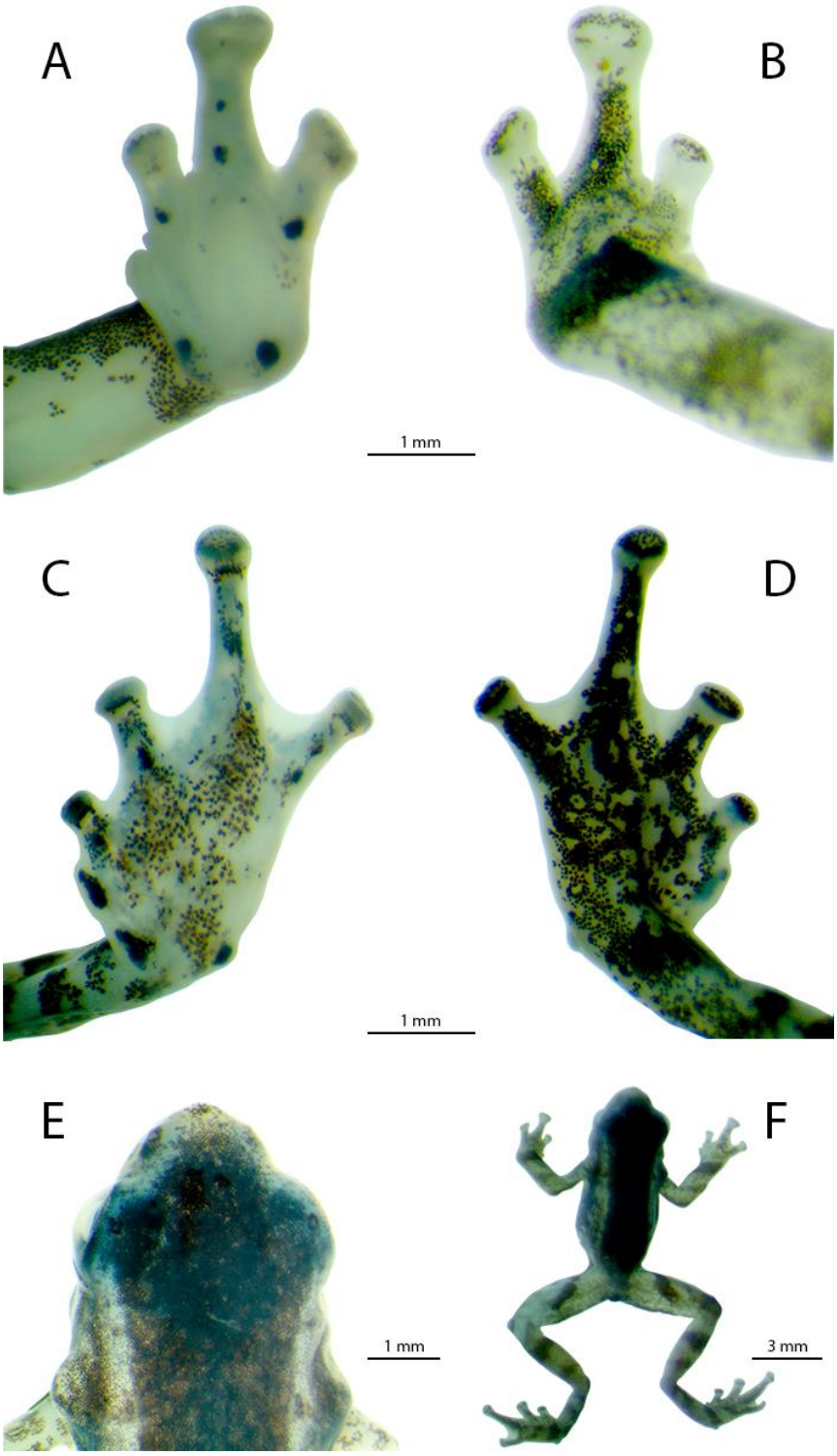


Fig 3. Preserved paratype HMOA 2165 from *Atopophrynus ikiam* sp. nov. Ventral and dorsal views of the hand (A-B) and foot (C-D), and dorsal view of head (E) and the body (F).

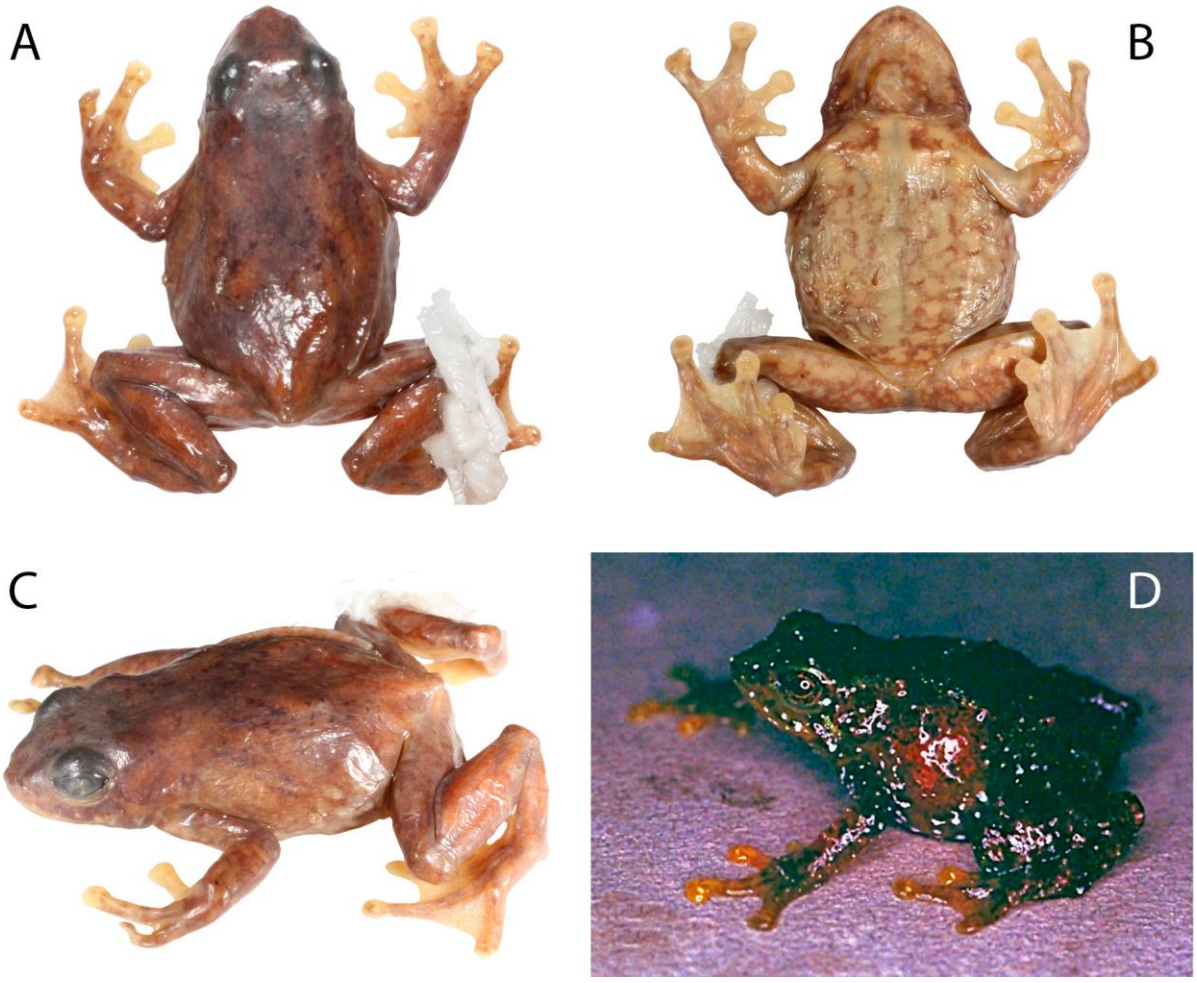


Fig 4. Holotype of *Atopophrynus syntomopus* from Cordillera Central, Departamento Antioquia, Colombia. (A-C) Preserved specimen (ICN 8611) reviewed at the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá. (D) Photo by J. D. Lynch.

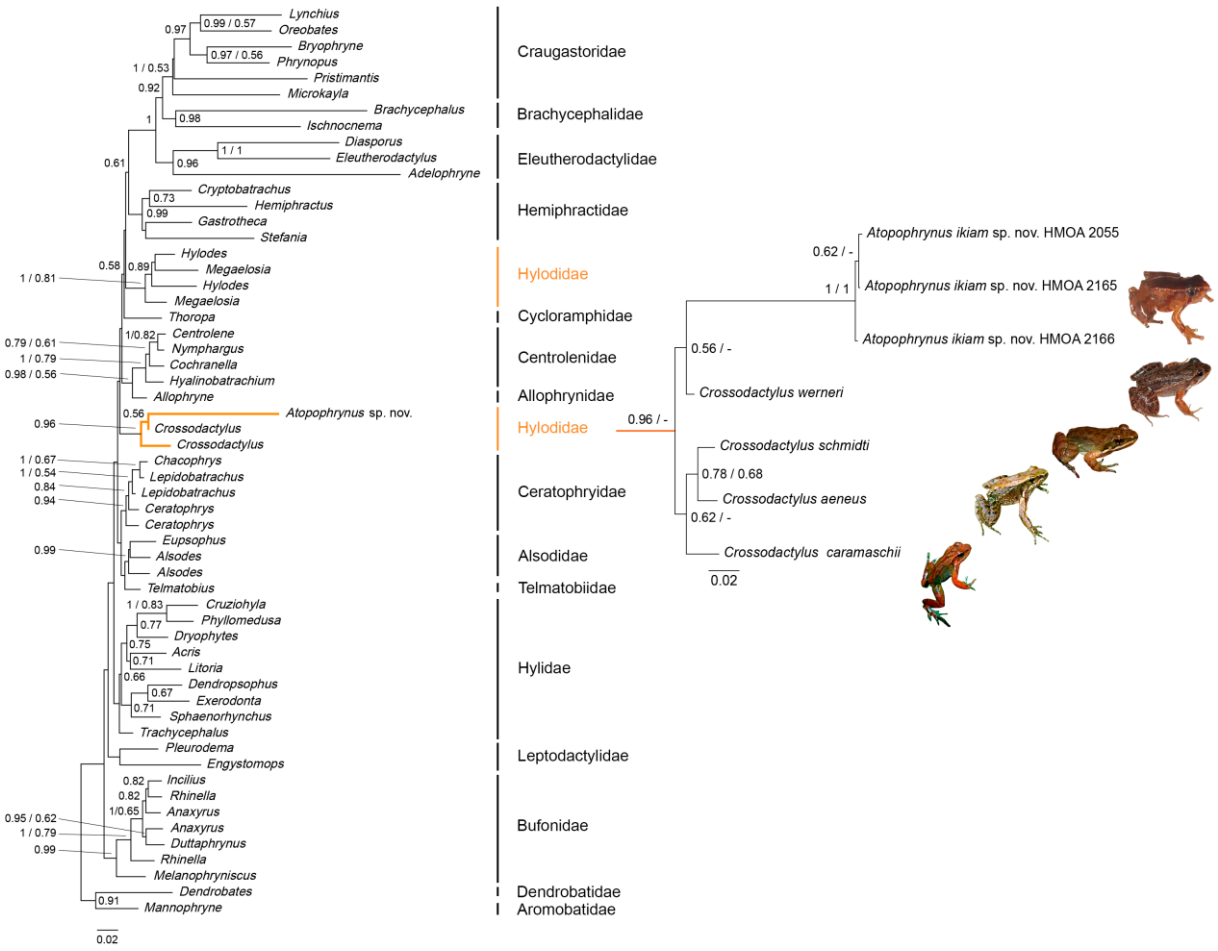


Fig 5. Phylogenetic relationships of Atopophrynus ikiam sp. nov. within the Hyloidea clade using sequences from two mitochondrial (12S and 16S) and one nuclear (RAG-1) genes. Support values are specified at nodes as Bayesian posterior probabilities/ML bootstrap. Values < 50 for ML support are not shown.

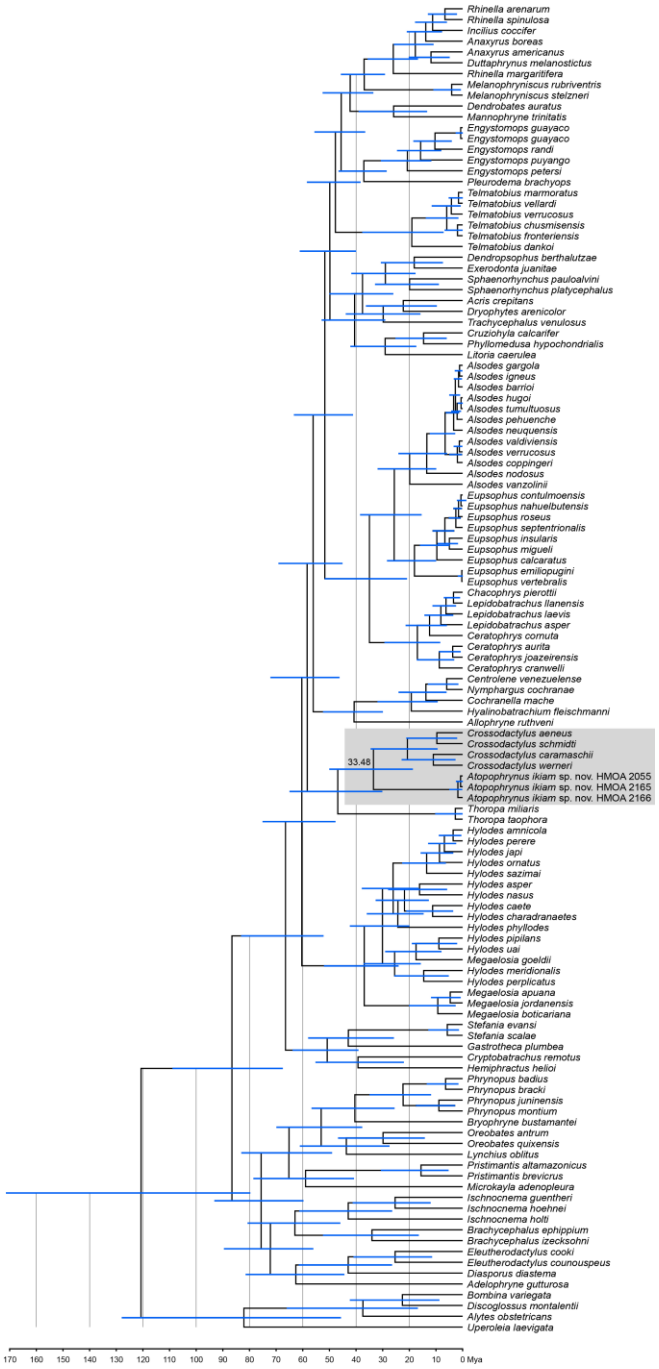


Fig 6. Outgroup + Hyloidea chronogram inferred by Beast. Node bars indicate the 95% highest posterior density according node ages. The calibration points used for Terrarana, Hemiphractidae, Centrolenidae + Allophrynidae, Leptodactylidae and Bufonidae groups are detailed in the methods.

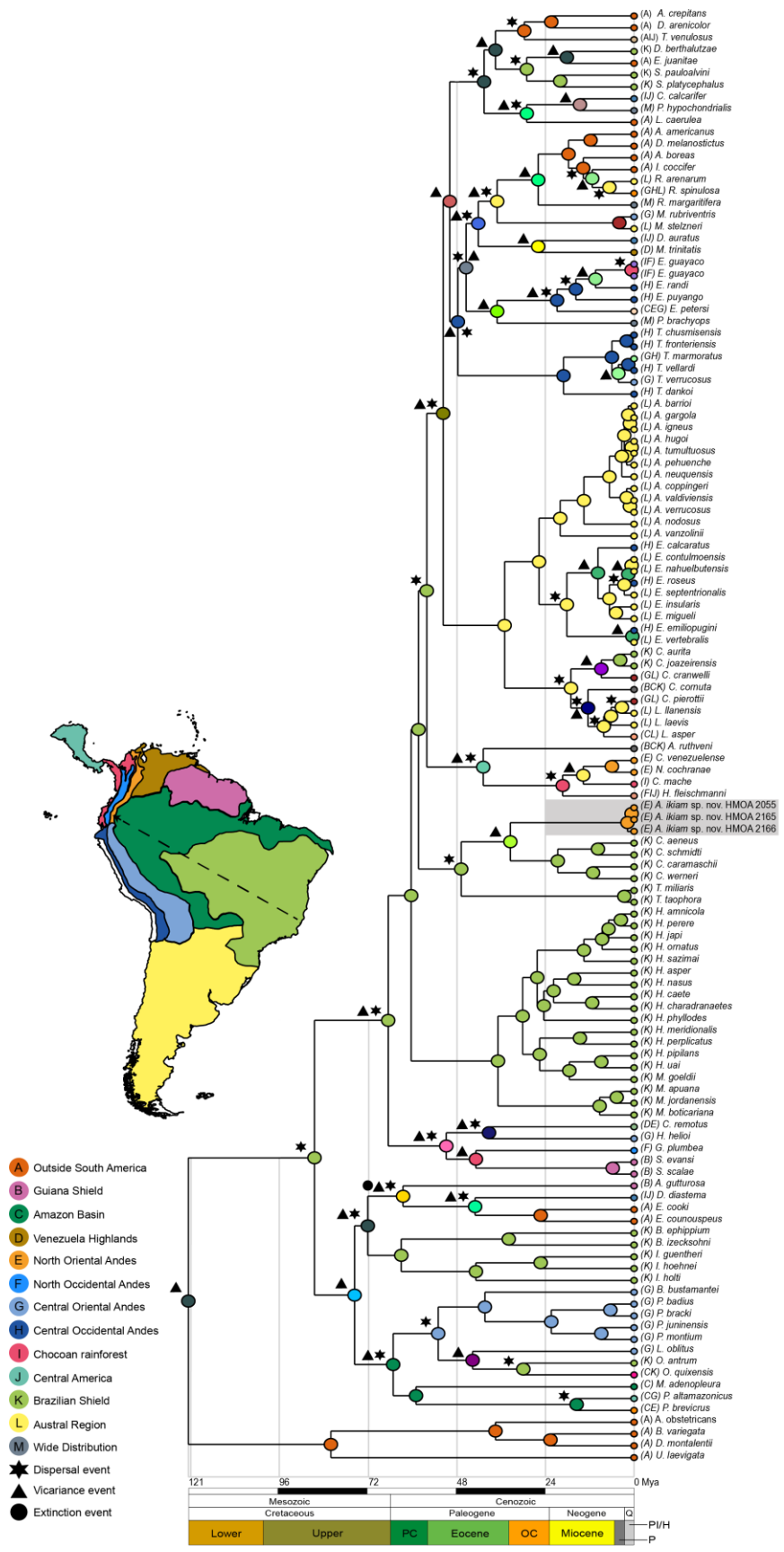
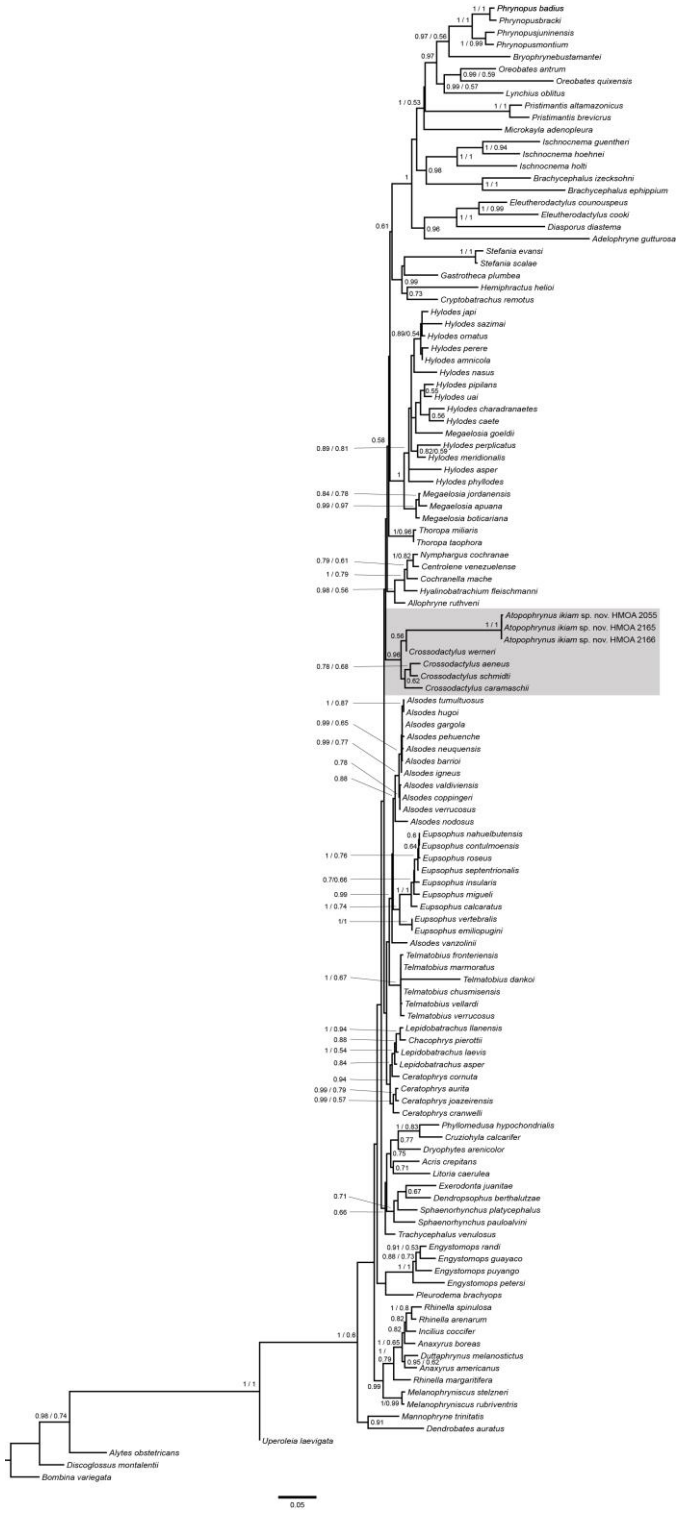

(A) A. crepitans
(A) D. arenicolor
(AIJ) T. venulosus
(K) D. berthalutzae
(A) E. juanitae
(K) S. pauloalvini
(K) S. platycephalus
(IJ) C. calcarifer
(M) P. hypochondrialis
(A) L. caerulea
(A) A. americanus
(A) D. melanostictus
(A) A. boreas
(A) I. coccifer
(L) R. arenarum
(GHL) R. spinulosa
(M) R. margaritifera
(G) M. rubriventris
(L) M. stelzneri
(IJ) D. auratus
(D) M. trinitatis
(IF) E. guayaco
(IF) E. guayaco
(H) E. randi
(H) E. puyango
(CEG) E. petersi
(M) P. brachyops
(H) T. chusmisensis
(H) T. fronteriensis
(GH) T. marmoratus
(H) T. vellardi
(G) T. verrucosus
(H) T. dankoi
(L) A. barrioi
(L) A. gargola
(L) A. igneus
(L) A. hugoi
(L) A. tumultuosus
(L) A. pehuenche
(L) A. neuquensis
(L) A. coppingeri
(L) A. valdiviensis
(L) A. verrucosus
(L) A. nodosus
(L) A. vanzolinii
(H) E. calcaratus
(L) E. contulmoensis
(L) E. nahuelbutensis
(H) E. roseus
(L) E. septentrionalis
(L) E. insularis
(L) E. migueli
(H) E. emiliopugini
(L) E. vertebralis
(K) C. aurita
(K) C. joazeirensis
(GL) C. cranwelli
(BCK) C. cornuta
(GL) C. pierottii
(L) L. llanensis
(L) L. laevis
(CL) L. asper
(BCK) A. ruthveni
(E) C. venezuelense
(E) N. cochranae
(I) C. mache
(FIJ) H. fleischmanni
(E) A. ikiam sp. nov. HMOA 2055
(E) A. ikiam sp. nov. HMOA 2165
(E) A. ikiam sp. nov. HMOA 2166
(K) C. aeneus
(K) C. schmidti
(K) C. caramaschii
(K) C. werneri
(K) T. miliaris
(K) T. taophora
(K) H. amnicola
(K) H. perere
(K) H. japi
(K) H. ornatus
(K) H. sazimai
(K) H. asper
(K) H. nasus
(K) H. caete
(K) H. charadranaetes
(K) H. phyllodes
(K) H. meridionalis
(K) H. perplicatus
(K) H. pipilans
(K) H. uai
(K) M. goeldii
(K) M. apuana
(K) M. jordanensis
(K) M. boticariana
(DE) C. remotus
(G) H. helioi
(F) G. plumbea
(B) S. evansi
(B) S. scalae
(B) A. gutturosa
(IJ) D. diastema
(A) E. cooki
(A) E. counouspeus
(K) B. ephippium
(K) B. izecksohni
(K) I. guentheri
(K) I. hoehnei
(K) I. holti
(G) B. bustamantei
(G) P. badius
(G) P. bracki
(G) P. juninensis
(G) P. montium
(G) L. oblitus
(K) O. antrum
(CK) O. quixensis
(C) M. adenopleura
(CG) P. altamazonicus
(CE) P. brevicrus
(A) A. obstetricans
(A) B. variegata
(A) D. montalentii
(A) U. laevigata
A Outside South America
B Guiana Shield
C Amazon Basin
D Venezuela Highlands
E North Oriental Andes
F North Occidental Andes
G Central Oriental Andes
H Central Occidental Andes
I Chocoan rainforest
J Central America
K Brazilian Shield
L Austral Region
M Wide Distribution
Dispersal event
Vicariance event
Extinction event
121
96
72
48
24
0 Mya
Mesozoic
Cenozoic
Cretaceous
Paleogene
Neogene
Q
Lower
Upper
PC
Eocene
OC
Miocene
PI/H
P

Fig 7. Ancestral area reconstruction from S-DIVA analysis inferred in RASP. Geographical areas: (A) Regions outside South America, related for outgroups; (B) Guiana Shield, (C) Amazon Basin, (D) Venezuelan Highlands, (E) North Oriental Andes, (F) North Occidental Andes, (G) Central Oriental Andes, (H) Central Occidental Andes, (I) Chocoan rainforest, (J) Central America, (K) Brazilian Shield, (L) Austral Region, (M) Wide distribution (more than three regions in South America). Abbreviations in the geological scale: Q = Quaternary, PI/H = Pleistocene/Holocene, P = Pliocene, OC = Oligocene, and PC = Paleocene.

Supporting information



S1 Fig. Phylogenetic relationships of *Atopophrynus ikiam* sp. nov. within Hyloidea clade (ingroup), by selected taxa using sequences from mitochondrial DNA (12S and 16S) and a nuclear gene (RAG-1). Bayesian posterior probabilities/ML bootstraps values are specified at nodes.