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Genome assembly of the endangered Santa Marta Harlequin Toad, *Atelopus laetissimus*, endemic to the Sierra Nevada de Santa Marta of Colombia

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Abstract

Despite the critical conservation status of many harlequin toads (Anura: Bufonidae: *Atelopus*) and their significance in elucidating the impacts of emerging diseases on amphibians, genomic data for this genus are scarce. Here, we present a de novo genome assembly of the Santa Marta Harlequin Toad, *Atelopus laetissimus*, using PacBio HiFi sequencing data, representing the first published *Atelopus* genome assembly and only the second amphibian genome assembly from Colombia. The assembly consists of 1,487 contigs at 35× coverage with an N50 of 31 Mb and a total length of 3.5 Gb. A Tetrapoda BUSCO score of 92% suggests our assembly is very nearly complete. Analysis revealed a high repeat content, with 70% of the genome consisting of interspersed repeats. This high-contiguity draft assembly will drive the search for a possible genetic basis of pathogen resistance in these endangered toads of the Sierra Nevada de Santa Marta, as well as facilitate ongoing evolutionary studies.

Key words: amphibians, biodiversity genomics, de novo genome assembly, Pacific biosciences

Introduction

The generation of high-quality reference genomes for amphibians has historically lagged behind other vertebrates due to the high costs and computational challenges associated with their often large, complex, and repetitive genomes (Rogers et al. 2018; Kosch et al. 2024). Recent technological advancements, including long-read sequencing and hybrid assembly approaches, have generated high-quality reference genomes (Rhie et al. 2021). However, much remains to be understood about the genetic basis of important natural history traits and structural composition of their genomes, including the evolution of transposable elements (TE), which are known to make up a significant portion of amphibian genomes (Hellsten et al. 2010).

Toads of the genus *Atelopus* (Anura: Bufonidae) are known as harlequin or stubfoot toads and are among the most endangered amphibians globally (Lötters et al. 2023). This decline is driven by a combination of threats, including habitat loss, climate change, and pollution, but the most devastating impact has been caused by chytridiomycosis, a fungal disease that has caused widespread amphibian declines (Valencia and Fonte 2021). Despite the critical status of many species of *Atelopus*, genomic resources for this genus remain limited. The scarcity of genomic data hinders our ability to better comprehend the molecular mechanisms underlying the resilience of species in the face of threats (Byrne et al. 2021; Paez et al. 2022). Consequently, investing in genomic research for amphibians holds immense potential for shedding light on the genetic basis of their response to infections and other threats (Kosch et al. 2019). Genomic resources serve as a safeguard for species existence, thereby informing targeted conservation efforts essential for this highly threatened genus (Blaxter et al. 2022).

While harlequin toads have declined drastically throughout the highlands of Central and South America, the five species of *Atelopus* found in the Sierra Nevada de Santa Marta (SNSM), a massive mountain on the Caribbean coast of Colombia,

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remain demographically stable. A major threat to amphibians worldwide is the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd), which has caused severe population declines and extinctions (Lips 2016). However, Bd has only been found at extremely low prevalence in the SNSM, suggesting that the pathogen has never entered an epizootic phase in the SNSM (Rueda-Solano et al. 2016). Thus, *Atelopus* in the SNSM may be uniquely resistant to the pathogen or simply have not experienced an outbreak of Bd.

Among harlequin toads of the Sierra, the best-studied species is *Atelopus laetissimus*, the Santa Marta Harlequin Toad, also known as the San Lorenzo Stubfoot Toad. Studies on this species have covered various organismal and ecological aspects, including reproduction (Rocha-Usuga et al. 2017; Rueda-Solano et al. 2022), behavior (Rueda-Solano and Warkentin 2016), and bioacoustics (Rueda-Solano et al. 2020). This species displays remarkable demographic stability and has even been reclassified at a lower threat level than previously, from Critically Endangered (CR) in 2004 to Endangered (EN) in 2014 due to new field observations and the lack of any clear declines in the SNSM (IUCN 2024). While the reasons for the persistence of *Atelopus* in the Sierra are not yet understood, based on studies of other amphibian species, potential factors might include a unique immune system in these species (distinct from the more susceptible species of *Atelopus*) or a powerful antifungal microbiota (as found in *A. elegans*; Flechas et al. 2012). On the other hand, persistence may be due simply to the continued absence of the fungal pathogen in the SNSM at levels capable of triggering an epizootic outbreak. Further genomic research on this species has the potential to elucidate any molecular mechanisms underlying its resilience and inform conservation strategies aimed at preserving the genus.

Here, we present the first draft de novo genome assembly of *Atelopus* using PacBio HiFi sequencing data and the first published *Atelopus* genome assembly. Additionally, we quantified the occurrence of a substantial number of repetitive segments. The reference genome of the Santa Marta Harlequin Toad will provide a valuable resource for studies on the diversification, evolution of morphological and physiological traits, susceptibility to diseases, and biological conservation of harlequin toads.

Methods

Field sampling and DNA extraction

Genomic data were generated from one adult male *A. laetissimus* from San Lorenzo, Magdalena Department, Colombia (Fig. 1), the type locality of this toad. The specimen was preserved under field collector number AJC 7668

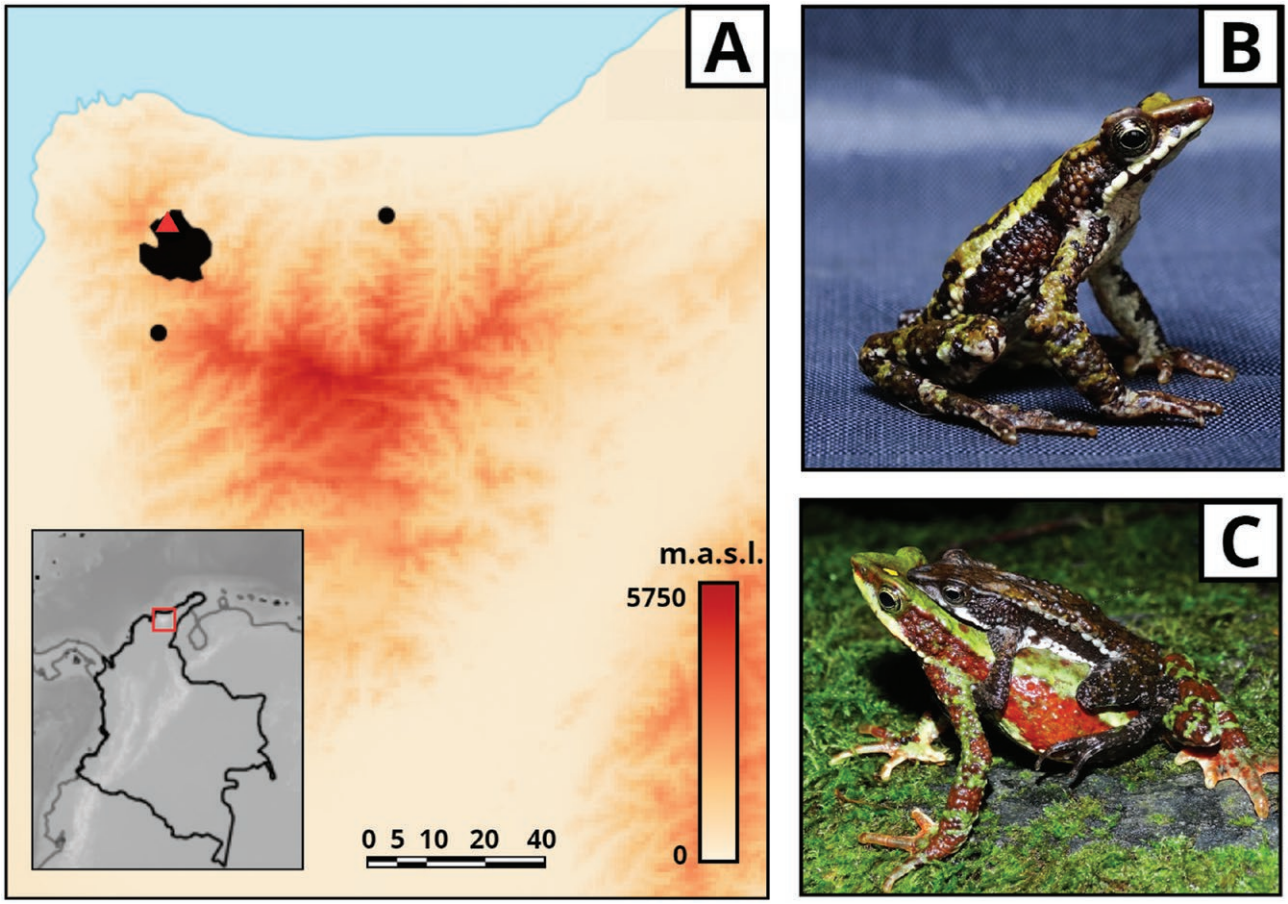

Fig. 1. The Santa Marta Harlequin Toad, *Atelopus laetissimus*, geographic distribution and phenotypes. a) Distribution of *A. laetissimus* in the SNSM: the red triangle represents the type locality and the source locality for the vouchered specimen. The black areas indicate other known localities. b) Specimen of *A. laetissimus* providing the tissue used to generate the reference genome, deposited in the Amphibian Collection of the *Museo de Historia Natural C.J. Marinkelle* at the Universidad de los Andes, Bogotá (field collector number AJC 7668). c) Amplexus of two individuals of *A. laetissimus* (not collected). Photos by LAR-S.

and deposited in the Amphibians Collection of the *Museo de Historia Natural C.J. Marinkelle* of the Universidad de los Andes. Liver and muscle tissue were snap-frozen in liquid nitrogen and stored immediately at −80 °C. Samples were shipped on dry ice to the DNA Sequencing Center at Brigham Young University (BYU) DNA Sequencing Center (Provo, Utah, USA) where genomic DNA was extracted and isolated from tissues using Qiagen Genome-tips according to the manufacturer's protocols.

Library preparation and sequencing

The SMRTbell library for HiFi sequencing was prepared at BYU after the instructions provided with the SMRTbell Express Template Prep Kit 2.0. High-molecular-weight genomic DNA was fragmented to achieve a desired size range of 15 to 18 kb. The fragmented DNA underwent concentration using 0.45× AMPure PB beads at 37 °C for 15 min to remove single-strand overhangs. Subsequently, the DNA underwent a set of enzymatic procedures. These steps encompass DNA damage repair at 37 °C for 30 min, followed by end repair and A-tailing at 20 °C for 10 min and 65 °C for 30 min. Ligation of Overhang Adapter v3 was carried out at 20 °C for 60 min and 65 °C for 10 min to inactivate the ligase. Lastly, the DNA underwent nuclease treatment at 37 °C for 1 h. The resulting SMRTbell library was purified and concentrated using 0.45× AMPure PB beads. Size-selection was performed using the BluePippin system to collect fragments larger than 10 kb. The final HiFi SMRTbell library, with an average fragment size of 16 kb, was sequenced using six 8M SMRT cells, Sequel II sequencing Chemistry 2.0, and 30-h movies for each cell on a PacBio Sequel II sequencer.

Genome assembly

A list of software used for the genome assembly is available in Table 1. Initially, we employed Cutadapt v. 4.3 (Martin 2011) to trim commonly utilized PacBio adapters. We performed *k*-mer counting ($k = 21$) and estimated genome size and heterozygosity as a preliminary evaluation of the genome using Jellyfish (Marçais and Kingsford 2011) and Genomescope2 (Ranallo-Benavidez et al. 2020). To generate the initial diploid assembly with the filtered HiFi reads, we used different assemblers: Hifiasm v. 0.19.5 (Cheng et al. 2021), HiCANU v. 2.1 (Nurk et al. 2020) and Improved Phased Assembler (IPA; Pacific Biosciences 2020). We also executed Hifiasm in the Galaxy platform (The Galaxy Community 2024).

We assessed the assembled genome statistics with QUAST 5 (Gurevich et al. 2013). To assess consensus quality (QV) and *k*-mer completeness, the fraction of reliable *k*-mers in the read set that are also found in the genome assembly, we used the Meryl database and Merqury (Rhie et al. 2020). To estimate genome completeness, we used BUSCO v. 5.5.0 which quantifies the presence of single-copy orthologs that should be highly conserved among all species (Manni et al. 2021). For the harlequin toad, we used the Tetrapoda ortholog database (tetrapoda_odb10.2024-01-08) which contains 5,310 genes.

We used BLAST v. 2.14.1 (Altschul et al. 1990) and MitoHifi v. 3.2.2 (Uliano-Silva et al. 2023) to search for a mitochondrial genome within our PacBio assembly of *A. laetissimus*. Searching for mitochondrial DNA sequence requires using a reference mitogenome from a related species. To choose the whole mitochondrial genome assembly most similar to *A. laetissimus* we used the tool findMitoReference.py available in MitoHiFi, which identified *Rhinella marina* (GenBank accession no. NC_066225.1) as the optimal reference.

Repeat annotation

To achieve a comprehensive characterization of the repetitive landscape within our assembly, we employed two distinct methodologies. First, we utilized RED 2.0, a novel de novo repeat discovery tool grounded in machine learning techniques (Girgis 2015). Second, we applied a high-precision de novo TE annotation pipeline, HiTE v. 3.2 (Hu et al. 2024), as well as RepeatModeler 2.0.6 (Flynn et al. 2020), for an in-depth annotation. Finally, we used the repeat library resulting from HiTE to mask the genome using RepeatMasker v. 4.1.5 (Smit et al. 2015).

Results

Sequencing data

Six cells of Sequel II generated the subreads that produced 8.3 million PacBio HiFi reads. These libraries yielded 35× coverage based on the Genomescope 2.0 genome size estimate of 3.25 Gb. The *k*-mer spectrum showed a bimodal distribution with two peaks, at ~14× and at ~33× coverage, where peaks correspond to heterozygous and homozygous states, respectively, of a diploid species (Fig. 2). We also estimated a 0.13% sequencing error rate with Genomescope 2.0.

We evaluated our three assemblies (Hifiasm, HiCANU, and IPA; Supplementary Table 1), and based on completeness (BUSCO score), contiguity (N50 statistics), and correctness (QV), we report the results of the Hifiasm assembly here (Fig. 2a). We obtained with Hifiasm a 3.5 Gb genome assembly (comprised of 1,487 contigs, with an N50 length of 31 Mb, minimum contig length of 13 kb, maximum contig length of 112 Mb, and mean coverage of 35× (matching the pre-assembly estimate from Genomescope; Fig. 2b). The QV score (phred-scaled error rate for the assembly) was 57.56, i.e. an estimated error rate of 0.0000017, while the *k*-mer completeness was 84.04%. With a BUSCO score of 91.7% of the 5,310 expected tetrapod single-copy orthologs being present in the assembly, we obtained a comprehensive representation of the *A. laetissimus* genome. Specifically, we recovered 4,870 complete BUSCOs of which 102 were duplicated,103 genes were fragmented, and 337 were missing from the assembly.

Repeat annotation

RED indicates that 65.44% or approximately 2.29 Gb of the *A. laetissimus* genome assembly consisted of interspersed repeats, while using HiTE with RepeatMasker, we were able to identify and mask 69.59% or 2.44 Gb of the genome assembly. DNA transposons constituted 16.95% of the genome, with hobo-Activator (8.96%) and Tc1-IS630-Pogo (7.01%) being the most abundant. Retroelements accounted for 8.5% of the assembly. Unclassified repeat elements made up 44.15% of the genome assembly. Additionally, small RNA, satellites, simple repeats, and low-complexity sequences were present in smaller proportions within the genome (0.1%). Using the RepeatModeler2 and RepeatMasker, we detected a slightly higher proportion of repetitive elements, with 70.62% (~2.48 Gb) of the genome masked. Within these repeats, retroelements comprised 23.91% (with LINEs at 5.65% and LTR elements at 18.25%), and DNA transposons made up

Table 1. Assembly pipeline and software used to assemble the genome of *Atelopus laetissimus*. Citations to the software are provided in the main text

	Software and options	Version
Filtering PacBio HiFi adapters	cutadapt -j 14 -e 0.1 --overlap 35 --revcomp --times 3 -b ATCTCTCTCAACAACAACAACGGAG GAGGAGGAAAAGAGAGAGAT -b ATCTCTCTCTTTTCCTCCTCCTCCGTTGTTGTTGTT GAGAGAGAT q.gz --discard-trimmed	4.3
k-mer counting	Jellyfish, Meryl	2.3.0, 1.3
Estimation of genome size and heterozygosity	GenomeScope	2.0
Average coverage assessment	samtools	1.12
De novo assembly (contiging)	Hifiasm -t 64 -f 37 -k 21 -w 51 -D 5.0 -N 100 -r 3 --max-kocc 20000 --primary	0.19.5-r587
Assembly statistics	quast	5.0.2
Assembly quality	Merqury	1.3
Completeness with single-copy orthologs	busco -m genome -l tetrapoda --tar --metaeuk_parameters "--split-memory-limit=350G --remove-tmp-files=1 --max-intron=1000000 --max-seq-len=3000000" --metaeuk_rerun_parameters" --in a_laetissimus.fa' --out busco_genome --cpu 4	5.5.0
De novo repeat annotation	HiTE: High-precision TE Annotator	2.0
De novo repeat annotation	RepeatModeler	2.0.6
Repeat masking	Repeatmasker	4.1.5
Repeat masking	RED	2.0
Quality assessment	BlobToolKit	4.3.0
Targeted mitochondrial reads assessment	makeblastdb -in R_marina_mitochondrion.fasta -dbtype nucl -out mito_db blastn -query a_laetissimus.fa -db mito_db -out mito_blast_results.txt -outfmt 6 -evalue 1e-5	2.14.1
Mitochondrial genome assembly	MitoHifi	3.2.2

12.59%, primarily driven by hobo-Activator (7.04%) and Tc1-IS630-Pogo (4.89%). According to RepeatModeler, unclassified elements comprised 33.21% of the genome assembly, whereas small RNA, simple repeats, and low-complexity sequences together constituted less than 1% of the genome (0.9%).

Mitochondrial DNA assembly

Using MitoHifi and BLAST we did not recover identifiable mitochondrial gene sequences from the assembly nor from the HiFi reads.

Discussion

Among bufonids, genome size estimates vary between 2.9 and 7.5 Gb (Liedtke et al. 2018; Gregory 2024; Kosch et al. 2024). Our *A. laetissimus* assembly falls closer to the bottom of this range with 3.5 Gb. Our assembly represents the fourth published genome of a bufonid and the first for *Atelopus* (Edwards et al. 2018; Lu et al. 2021; Streicher et al. 2021). Despite their conservation status, genomic resources for *Atelopus* remain scarce, due in part to the extreme difficulty in obtaining fresh tissues from most of these highly declined species. Byrne et al. (2021) employed whole exome sequencing of *A. zeteki* and *A. varius* from Panama to explore the genetic underpinnings of persistence in the face of Bd, illustrating how -omics data can be used to understand amphibian declines.

Characterizing repetitive elements is hindered by a lack of amphibian-specific annotations and other genomic data. Based on the *RepeatMasker* database, we find that the genome assembly of *A. laetissimus* reveals large expansions and accumulations of repetitive elements. Notably, 70% of its assembly is characterized by repetitive elements, and a large portion of the total assembly (at least 33%) consists of unknown and undescribed elements, highlighting the need for further investigation into the diversity and distribution of TEs and other annotation tools for amphibians. *Xenopus tropicalis*, for example, has approximately one-third of its genome derived from TEs, with DNA transposons comprising about three-quarters of this TE content (Hellsten et al. 2010; Chalopin et al. 2015; Bredeson et al. 2024). Other anuran species also exhibit a high proportion of repetitive elements, such as *Oophaga pumilio*, whose genome is comprised of 70% repeats (Rogers et al. 2018), similar to *A. laetissimus*, whose repeat content is comparable to that of larger amphibian genomes, such as *Dendrobates tinctorius* (Dittrich et al. 2024) and *Ranitomeya imitator* (Stuckert et al. 2024), indicating potential commonalities in the evolutionary dynamics of TEs, though differences in assembly and annotation methods may explain some of this variation.

Our genome assembly of *A. laetissimus* is one of the most complete genome assemblies among amphibians (Kosch et al. 2024). This genome assembly not only enhances our understanding of the repetitive elements but provides a crucial resource for future comparative genomics studies. Despite the completeness of the nuclear genome, we were unable to obtain the mitochondrial genome. The lack of mitochondrial sequence data was possibly caused by the size-selection during the HiFi SMRTbell library preparation. This step could have filtered out reads reducing mitochondrial reads coverage for consensus calling.

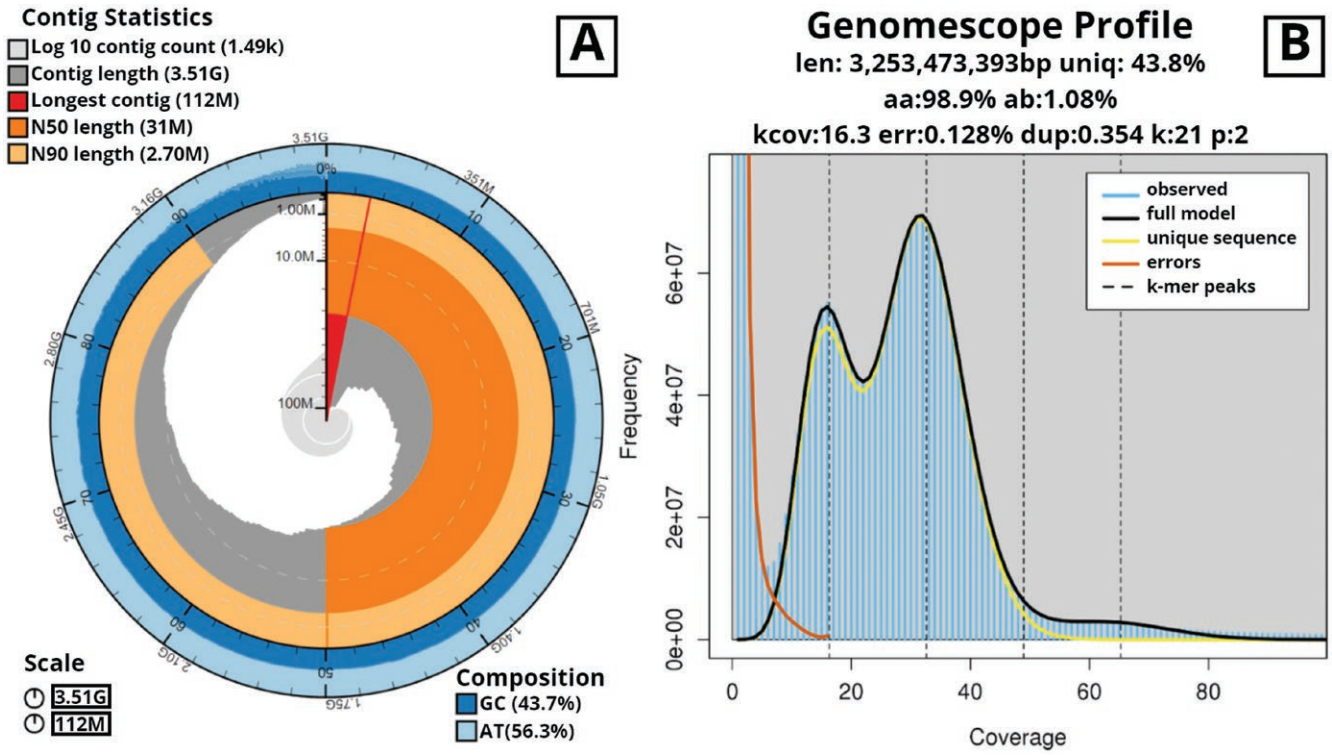

Fig. 2. Genome assembly statistics. a) BlobToolKit Snail Plot illustrating the quality metrics for the primary Hifiasm assembly of the *A. laetissimus* genome. The statistics are described on The plot represents the entire assembly, with the central plot focusing on length-related metrics. The red line indicates the length of the longest contig, and the remaining contigs are depicted in gray and arranged in decreasing size order clockwise around the central plot. Dark and light orange arcs represent the contig N50 and contig N90 values. The central light gray spiral depicts the cumulative contig count, with white lines marking each order of magnitude. Dark and light blue areas on the outside indicate mean, maximum, and minimum GC versus AT content at 0.1% intervals. Statistics related to this panel are provided in Supplementary Table 1. b) *k*-mer spectrum output derived from PacBio HiFi data, excluding adapters, utilizing GenomeScope 2.0. *k*-mers at lower coverage and frequency indicate divergence between haplotypes, while those at higher coverage and frequency signify similarity between haplotypes.

Understanding the genomic basis of amphibians is critical not only for comprehending the mechanisms of their evolution but also for developing effective conservation strategies to mitigate the threats they face and ensure their survival for future generations (Blaxter et al. 2022). De novo genome assemblies provide resources for studying ecology and evolution, and for guiding conservation efforts aimed at preserving this highly endangered genus (Shaffer et al. 2015). Genomic data can identify important population traits, such as effective population size, inbreeding, demographic history, and population structure, which can inform ongoing breeding programs and other conservation strategies (Paez et al. 2022; Talavera et al. 2024). Comparative and population genomics of immune gene diversity in susceptible versus resistant species should provide insights into causes, consequences, and possible interventions to combat disease in amphibians (Funk et al. 2018, Trumbo et al. 2023). Additionally, the availability of a high-quality genome assembly makes possible comparative genomics studies within the Bufonidae family, shedding light on the evolutionary history and adaptive traits of these amphibians.

Supplementary material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/ online.

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Author contributions

Victor Nascimento de Araújo (Data curation, Investigation, Project administration, Writing—original draft), Jose Grau (Data curation, Formal analysis, Writing—review & editing), Luis Rueda-Solano (Funding acquisition, Investigation, Writing—review & editing), Jose Barros-Castañeda (Investigation, Writing—review & editing), Sandra Flechas (Funding acquisition, Writing—review & editing), and Andrew Crawford (Funding acquisition, Supervision, Writing—review & editing)

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Conflict of interest statement. None declared.

Data availability

Data generated in this study are available in the NCBI BioProject repository under accession PRJNA1142550. Sequencing data (BioSample SAMN42946352) are deposited in the NCBI Sequence Read Archive (SRA) under experiment accessions SRR30177483, SRR30177484, SRR30177485, SRR30177486, SRR30177487, and SRR30177488 (PacBio HiFi sequencing data). The final genome assembly file has been deposited in the NCBI WGS database under the accession number JBGDNC00000000.

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