Multi-omics resources for the Australian southern stuttering frog (*Mixophyes australis*)

reveal assorted antimicrobial peptides

Simon Tang[1,2], Emma Peel[1,2], Katherine Belov[1,2], Carolyn J. Hogg[1,2,†], Katherine A.

Farquharson[1,2]

[1] School of Life and Environmental Sciences, The University of Sydney, NSW, 2006,

Australia

[2] Australian Research Council Centre of Excellence for Innovations in Peptide and Protein

Science, The University of Sydney, NSW 2006, Australia

[†] Corresponding Author: carolyn.hogg@sydney.edu.au

Running title: Stuttering frog AMP discovery

ABSTRACT

The number of genome-level resources for non-model species continues to rapidly expand. However, frog species remain underrepresented, with up to 90% of frog genera having no genomic or transcriptomic data. Here, we assemble the first genomic and transcriptomic resources for the recently described southern stuttering frog (*Mixophyes australis*). The southern stuttering frog is ground-dwelling, inhabiting naturally vegetated riverbanks in south-eastern Australia. Using PacBio HiFi long-read sequencing and Hi-C scaffolding, we generated a high-quality genome assembly, with a scaffold N50 of 369.3 Mb and 95.1% of the genome contained in twelve scaffolds. Using this assembly, we identified the mitochondrial genome, and assembled six tissue-specific transcriptomes. We also bioinformatically characterised novel sequences of two families of antimicrobial peptides (AMPs) in the southern stuttering frog, the cathelicidins and β-defensins. While traditional peptidomic approaches to peptide discovery have typically identified one or two AMPs in a frog species from skin secretions, our bioinformatic approach discovered 12 cathelicidins and two β-defensins that were expressed in a range of tissues. We investigated the novelty of the peptides and found diverse predicted activities. Our bioinformatic approach highlights the benefits of multi-omics resources in peptide discovery and contributes valuable genomic resources in an under-represented taxon.

INTRODUCTION

Since the completion of the Human Genome Project in 2003, there has been a proliferation of high quality genomic and transcriptomic resources for non-model species (Hotaling et al. 2021; Lewin et al. 2022; Lewin et al. 2018). The ability to generate these 'multi-omics' resources in diverse species has clear comparative (Horgan and Kenny 2011), conservation (Formenti et al. 2022; Paez et al. 2022), and clinical benefits (Wong et al. 2021). As of July 2023, over 5,800 non-model animal genomes and 4,600 non-model animal transcriptomes are available in the largest global genome repository, the National Center for Biotechnology Information (NCBI) (Sayers et al. 2022). Despite the acceleration of genomic resources, only 46 genera within the Order Anura (10% of all frog

genera) have genomic or transcriptomic resources available on NCBI (AmphibiaWeb 2022; Sayers et

al. 2022). In Australia, despite 92% of frogs being endemic, only 4 of the 229 known endemic frog

species currently have 'omics level resources available (AmphibiaWeb 2022; Farquharson et al. 2023;

Lamichhaney et al. 2021; Li et al. 2020; Rhie et al. 2021). The global disparity in genetic resources

across taxa has been driven in part by the diverse structures of amphibian genomes and associated

difficulties with sequencing and assembly (Lamichhaney et al. 2021; Smith et al. 2019). Frogs have

highly variable genome sizes, (Lamichhaney et al. 2021; Liedtke et al. 2018); variable proportions of

repetitive elements (Li et al. 2020; Seidl et al. 2019); and can have ploidy variation (Novikova et al.

2020).

An emerging application of genomic resources is the bioinformatic discovery of novel antimicrobial

peptides (AMPs). AMPs are small peptides found across all classes of life that form an important part

of the innate immune response (Hanson et al. 2019; Huan et al. 2020; Wang et al. 2016). The

comprehensive characterisation of AMPs is valuable in understanding how different species respond

to pathogens in their environment and their pleiotropic immune effects have also been explored as

potential therapeutics, with several currently undergoing clinical trials (Mercer et al. 2020; Ridyard

and Overhage 2021). Frogs are known to express AMPs from the exocrine glands of their skin in

response to their exposure to diverse, microbial communities in their amphibious, freshwater habitats

(Ladram and Nicolas 2016; Varga et al. 2018; Wang et al. 2020). Over 2,500 novel AMPs have been

characterised from only 167 frog species on the Database of Anuran Defence Peptides v1.6 (DADP)

(Novković et al. 2012). To date, frog AMPs have typically been characterised with a peptidomic

approach; the iterative purification of AMPs showing antimicrobial activity from frog skin secretions

using high-performance liquid chromatography (HPLC) (Yang et al. 2022). However, this method

may not capture the suite of AMPs expressed in other tissues (Hao et al. 2012), nor AMPs with

immune functions other than antimicrobial activity (He et al. 2019). In response, there has been

increased interest in 'mining' genomic and transcriptomic resources for the genes that encode for

AMPs (Peel et al. 2016). These methods are primarily homology-driven, using known sequences of

large, well-characterised AMP families like cathelicidins and β-defensins to search for homologs

(Dalla Valle et al. 2012). A genomics-driven approach has been applied to discover novel AMPs from

a range of non-model species (Peel et al. 2016; Pérez de la Lastra et al. 2021; Wang et al. 2021; Yoo

et al. 2015). However, despite the evolutionary divergence of Australian frogs, none have yet been

investigated for AMPs using a bioinformatic approach.

The southern stuttering frog (*Mixophyes australis*) (Mahony et al. 2023) is a small, ground-dwelling

frog inhabiting naturally vegetated riverbanks in south-eastern Australia, and is part of the

Myobatrachidae family of endemic Australian ground frogs (Barker et al. 1995; Cogger 2000). The

northern (*Mixophyes balbus*) and southern (*Mixophyes australis*) species of stuttering frog were once

considered a single species, but were recently split (Mahony et al. 2023). Although yet to be officially

assessed, application of the International Union for the Conservation of Nature (IUCN) threat

assessment methods for the southern stuttering frog warrants a listing of 'Endangered' for this newly

defined species (Mahony et al. 2023). As a ground-dwelling frog, southern stuttering frogs spend most

of their life cycle exposed to large bodies of water and decomposing detritus, which may carry

pathogens such as chytrid fungus (*Batrachochytrium dendrobatidis*) and Ranaviruses (Mahony et al.

2023; Murray and Hose 2005). We hypothesise that the selective pressures of the southern stuttering

frog's environment make it a good candidate for the discovery of novel and diverse AMPs.

Preliminary studies on other *Mixophyes* species demonstrated some resistance against chytrid fungus

in skin secretions (Hollanders et al. 2023; Woodhams et al. 2007). While these secretions have not

been sequenced, these studies suggest possible AMP activity in the *Mixophyes* genus.

In this study, we use a combination of PacBio HiFi long-read, short-read, and Hi-C sequencing data to

generate the first genomic and transcriptomic resources for the southern stuttering frog. Using these

high-quality resources, we use bioinformatics to characterise and analyse the AMP families

cathelicidins and β-defensins. By generating the first 'omics level resources for the *Mixophyes* genus,

this study provides a unique opportunity to investigate an otherwise largely understudied, Australian

taxon.

RESULTS

Multi-omics resources for the southern stuttering frog

Using a combination of PacBio HiFi long-read and Hi-C sequencing data, we generated a high-quality

genome assembly for the southern stuttering frog (Figure 1A). The genome is 3.13 Gbp in length, has

a read coverage of 26× and a scaffold N50 of 369 Mbp (Table 1). Repeat-masking the genome

revealed a high proportion of repetitive elements (53.7%; Table S1), within the broad expected range

for Anuran genomes (32.0-77.1%; (Sayers et al. 2022)). The GC content of the genome, 40.3%, was

also within the range of expected Anuran GC values (26.9-44.5%; (Sayers et al. 2022)). From the

assembly, we identified a scaffold representing the mitochondrial genome, which contained 36 genes:

21 tRNAs, 2 rRNAs and 13 protein-coding genes (Figure 1B).

Figure 1. A) The 12 longest southern stuttering frog genome scaffolds, ordered by length. Red dots

indicate the presence of telomeric sequences at the end of scaffolds. Horizontal, black lines indicate

contig joins informed by the Hi-C sequencing data. **B)** Circos plot of the southern stuttering frog

mitochondrial genome generated using Proksee (Grant et al. 2023). Outer ring contains genes on the

forward strand, while the inner ring contains genes on the reverse strand.

Over 95% of the genome was assembled in twelve scaffolds (Figure 1A; Figure S1), which likely

represent the chromosomes based on previous karyotypic analysis of other *Mixophyes* species

(2n=24), including *M. hihihorlo* (Donnellan et al. 1990), *M. fasciolatus* and *M. schevilli* (Schmid et al.

2002). Six scaffolds were flanked at each end by long, telomere-like repeats, while four scaffolds had

telomere-like repeats on a single end (Figure 1A). Additionally, a substantial drop in scaffold length

between the 12th and 13th scaffold (94.13Mbp vs 4.75Mbp) was noted, further suggesting 12

chromosome-level scaffolds.

To compare the contiguity and completeness of the genome to other frog genomes, we calculated

several genomic statistics, including benchmarking universal single-copy orthologs (BUSCO) (Simão

et al. 2015) using the vertebrata_odb10 lineage. Genome statistics were calculated for two model frog

species and the three publicly available scaffolded Australian frog genomes (Table 1). The southern

stuttering frog had a similar scaffold L50 (L50 = 5) to other recent high-quality genome assemblies

such as *Pseudophryne corroboree* (L50 = 4). Our BUSCO analysis revealed that our assembly

contained 91.8% of complete BUSCOs, 2.9% were present but fragmented, while 5.3% were missing.

The proportion of complete BUSCOs in the southern stuttering frog is slightly lower, but still

comparable to the model reference *Xenopus laevis* (95.9%). An unbiased assessment of whole

genome completeness with merqury (Rhie et al. 2020) showed that our genome assembly was highly

complete (93.5%) and accurate (Q60+).

Using Illumina short-read RNA sequencing, we generated six reference-guided, tissue-specific

transcriptome assemblies (dorsal skin, ventral skin, liver, spleen, brain, and gonads) that were aligned

into a global transcriptome. All tissues had high mapping rates to the genome (78.62-89.37%), with

both the tissue-specific and global transcriptomes yielding high complete BUSCO scores (84.0-

96.1%; Table S2). A total of 36,540 genes were annotated using FGENESH++ (Solovyev et al. 2006).

Of these, 16,355 were annotated using evidence from the coding regions of the global transcriptome,

similar to the 16,279 predicted coding regions provided as input. A further 12,892 genes were

annotated via homology to non-redundant, metazoan, proteins from NCBI, and 7,293 were annotated

ab initio. Proteins from these annotated genes yielded a complete BUSCO score of 86.0%,

comparable to the genome annotation of *Limnodynastes dumerilii*, and lower than the *Xenopus* spp.

annotations (Table S2). However, a large proportion of the stuttering frog annotated genes were

fragmented (8.5%), similar to *L. dumerilii* (8.0%).

Table 1. Statistics generated from the genome and transcriptomes of the southern stuttering frog
(*Mixophyes australis*; this study), the model *Xenopus* species and three Australian frog genome
assemblies. If available, the NCBI RefSeq or GenBank assembly number is provided. Read alignment
rate refers to the percentage of tissue-specific RNA reads that mapped to the genome. BUSCO v5.3.2
(Simão et al. 2015) scores were calculated in this study for all genomes and transcriptomes using the
vertebrata_odb10 lineage. There was no publicly available transcriptome for *L. dumerilii, P. ornatum*
and *P. corroboree*, so transcriptome statistics could not be generated for these species. Full BUSCO
scores for the genomes, transcriptomes and gene annotations are provided in Table S2.

Genome Statistics						
Species and Assembly Number	**Size (Gb)**	**Contig No.**	**Contig L/N50 (Mb)**	**Scaffold No.**	**Scaffold L/N50 (Mb)**	**Complete BUSCOs (%)**
Southern stuttering frog (*Mixophyes australis*)	3.120	1,290	85/11.04	790	4/369.31	91.3
African clawed frog (*Xenopus laevis*) [GCF_017654675.1]	2.742	716	35/22.45	55	8/155.25	95.9
Western clawed frog (*Xenopus tropicalis*) [GCF_000004195.4]	1.451	851	32/14.63	167	5/153.96	95.3
Southern banjo frog (*Limnodynastes. dumerilii*) [GCA_011038615.1]	2.379	739,331	58,116/0.01	520,896	2,127/0.29	80.9
Ornate burrowing frog (*Platyplectrum ornatum*) [GCA_016617825.1]	1.065	238,568	39,409/0.004	148,035	9,283/0.03	42.4
Corroboree frog (*Pseudophryne corroboree*) [GCA_028390025.1]	8.873	5,826	353/6.82	3,127	5/846.89	93.1
Transcriptome Statistics						
Species	**Type**	**Alignment Rate (%)**		**No. Transcripts**	**Complete BUSCOs (%)**	
Southern stuttering frog (*M. australis*)	Brain	86.23		66,201	89.9	
	Gonads (male)	78.62		88,716	93.0	
	Ventral skin	88.32		52,141	89.3	
	Dorsal skin	87.96		55,849	89.8	
	Liver	89.37		46,579	84.0	
	Spleen	83.62		60,664	90.2	
	Global	N/A		192,115	96.1	
African clawed frog (*X. laevis*) [GJQV00000000.1]	Follicle	N/A		333,966	70.8	
Western clawed frog (*X. tropicalis*) [GIVH00000000.1]	Gonads (male and female)	N/A		372,630	95.7	

Characterisation of cathelicidins and β-defensins

We used the genome and transcriptome assemblies of the southern stuttering frog to characterise two families of AMPs: cathelicidins and β-defensins. These families were selected due to their conservation across vertebrate species (Kosciuczuk et al. 2012; Tu et al. 2015), highly conserved structures and motifs (Chen et al. 2021; Shen et al. 2016; Yu et al. 2022) and demonstrated antimicrobial potency (Shen et al. 2016; Wei et al. 2015). We identified 12 cathelicidin (MA-CATH1-12) and two β-defensin (MA-BD1 and MA-BD2) genes in the genome using homology-based search strategies. Full-length transcripts of all cathelicidins and β-defensins were identified in the global transcriptome. All putative AMPs contained the characteristic features of each family, including expected exon number (four for cathelicidins and two for β-defensins), conserved amino acid residues, motifs, and domain structure (Figure S2 and S3). As expected, each AMP family was encoded in clusters within the genome; all cathelicidins were located on scaffold 5 and all β-defensins on scaffold 2 (Figure S4). Cathelicidin and β-defensin genes were named in the order in which they are encoded within the genome (Table S3; Figure S4). Amino acid sequences of all AMPs identified in this study, with predicted signal peptide and mature peptide domains are provided in Figures S2 and S3.

Properties, evolutionary relationships and expression patterns of cathelicidins and β-defensins

The mature peptide domains of cathelicidins and β-defensins are the bioactive, antimicrobial portion of the peptide. In the southern stuttering frog, the mature peptides of the cathelicidins ranged between 10-161 amino acids in length (Figure S2), while the two β-defensins were both 47 amino acids (Figure S3). The mature peptides of AMPs generally have a high positive charge which facilitates electrostatic interaction and attachment to microbial cell membranes (Huan et al. 2020). Stuttering frog cathelicidins MA-CATH1, 2 and 3 had a high cationic charge of 8.9, 4.9 and 27.3 respectively at pH 7 (Table S4). However, several AMPs identified in this study were weakly cationic (charge 0-3), and MA-CATH8 and MA-CATH2 were anionic. Similarly, while a large proportion (10/14) of the characterised AMPs had >30% hydrophobic residues, another general trend of AMPs (Chen and Lu 2020), some peptides like MA-CATH3 had as few as 12.42% hydrophobic residues (Table S4).

Amphipathicity is often seen in AMPs to permeabilise microbial membranes (Chen and Lu 2020).

Through visual inspection of Kyte and Dolittle hydropathicity plots, almost all the southern stuttering

frog AMPs exhibited amphipathicity, with the N-terminus of the peptides being generally more

hydrophilic than the C-terminus (Figure S5A, C). MA-CATH3 exhibited some regions of

hydrophobicity, but was largely hydrophilic (Figure S5B).

As the first AMPs characterised in the *Mixophyes* genus, we explored their sequence diversity and

relationship to other known frog AMPs. All stuttering frog AMPs displayed low percent identity to

other known AMPs by BLAST, with MA-BD2 having the highest percent identity of all AMPs in this

study to a Chinese spiny frog (*Quasipaa spinosa*) β-defensin (64.15%) (Table S4). As expected,

maximum likelihood phylogenetic trees generated using all known frog cathelicidins and β-defensins

reflected this diversity, as indicated by the long branch lengths (Figure 2). MA-CATH1-3 and MA-

CATH4-8 formed two species-specific clades within the cathelicidin tree, some of which were

strongly supported with >95% ultrafast bootstrap support (Figure 2). MA-CATH9-12 clustered within

clades containing AMPs from multiple Asian and African frog species, albeit with low bootstrap

support (Figure 2). While Figure 3 suggests that the southern stuttering frog β-defensins are more

closely related than the other β-defensins, this relationship was not well supported.

Our comprehensive bioinformatic AMP discovery approach allowed us to explore the expression of

AMPs across a range of tissues beyond skin, which is typically the target of peptidomic approaches to

AMP discovery. As expected, we observed high AMP gene expression in the dorsal and ventral skin.

However, we also observed AMP gene expression in other internal organs (Figure 4). AMP gene

expression was found in the liver, spleen, and gonads, with MA-CATH6 and 8 showing the highest

expression. MA-CATH10 and MA-BD2 exhibited higher expression in the skin than other AMPs,

while some peptides like MA-CATH12 were lowly expressed across all the tissues when compared to

other AMPs (Figure 4).

Figure 2. Phylogenetic relationships amongst frog cathelicidins. Tree was generated using the maximum likelihood method, and rooted using four fish cathelicidins as an outgroup. Branches are coloured by ultrafast bootstrap values, with values >95% in red, and the remaining branches coloured in black. Stuttering frog cathelicidins are coloured green. Cathelicidins are labelled by geographic region; Oceania is green; Asia is blue; North America is pink; Africa is yellow; and Asia and Europe combined is purple. The tree was annotated using MEGA11. For frog cathelicidin sequences used, see Table S5.

217 **Figure 3.** Phylogenetic relationships amongst frog defensins. Tree was generated using the maximum

218 likelihood method and is unrooted. Branches are coloured by ultrafast bootstrap values, with values

219 >95% in red, and the remaining branches coloured in black. Stuttering frog defensins are coloured

220 green. Defensin are labelled by geographic region; Oceania is green and Asia is blue. The tree was

221 annotated using MEGA11. For frog defensin sequences used, see Table S5.

Figure 4. Expression data of the novel cathelicidins (MA-CATH1-12) and β-defensins (MA-BD1-2) from the stuttering frog. Transcripts per Million (TPM) values were derived from the tissue-specific transcriptomes. Note different x-axis scales due to differences in relative expression of AMPs between tissues.

DISCUSSION

Here, we used a combination of PacBio HiFi and Hi-C data to generate the first contiguous, annotated, reference genome for the southern stuttering frog. In addition, we assembled the mitochondrial genome and six tissue-specific transcriptomes that were merged into a global transcriptome. The level of resources generated for this single species is comparable with model *Xenopus* species, as few frogs have a publicly available nuclear genome, mitochondrial genome, as well as transcriptome. Additionally, they are the first 'omics level resources in the *Mixophyes* genus. As the Myobatrachidae family is one of the oldest, most diverse frog families in Australia (Brennan et al. 2023), these resources add to our understanding of Australian fauna.

To demonstrate the insights that can be gained from these genomic and transcriptomic resources, we bioinformatically characterised 12 cathelicidins and two β-defensins, the first Australian frog AMPs to be discovered using this approach. While other peptidomics-based studies describing cathelicidins in frogs have revealed at most two per species (Ling et al. 2014; Yu et al. 2013), 12 cathelicidins were discovered in this study. Similarly, while one β-defensin has been characterised per species in frogs (Shen et al. 2016; Yu et al. 2022), two were found in this study. The expression analysis from the transcriptomic data revealed some of these AMPs, such as MA-CATH1, MA-CATH10 and MA-BD2, were primarily expressed in the dorsal and ventral skin. However, several others had little to no expression in the skin but were primarily expressed in the liver (MA-CATH6), spleen (MA-CATH7-8), and low levels in the brain (MA-BD1). The peptides that were not expressed in the skin would not have been identified if the more conventional, peptidomics approach of screening skin secretions was used. Our results reveal the benefits of a genomic-based AMP discovery approach and the need for more amphibian genomic resources to characterise such peptides.

It is possible that the evolutionary isolation of Australian amphibians compared to other characterised species has resulted in the diverse suite of cathelicidins and β-defensins observed. Alternatively, the range of cathelicidins and β-defensins characterised here may suggest that the southern stuttering frog is under stronger microbial exposure than other frogs. A previous study on *Diptera* (fly) species has shown that AMP diversity and gene duplication is positively correlated with microbial exposure

(Hanson et al. 2019). This is also likely the case in frogs as they adapt to different global aquatic and

terrestrial environments. It is more likely, however, that other frog species and genera may also

exhibit a similar number and diversity of cathelicidins and β-defensins which are uncharacterised. The

lack of bioinformatic AMP discovery for most frog species makes comparative analysis difficult.

Future investigations into frog AMP diversity that incorporate a genomic-based discovery platform

will facilitate direct comparisons between species.

Our phylogenetic analysis revealed that some southern stuttering frog cathelicidins formed species-

specific sister clades to those containing cathelicidins from frogs of Europe, North America, and Asia

(Figure 2). This may indicate that these particular cathelicidins were the result of gene duplication

events that occurred after the southern stuttering frogs diverged from the other frog species within the

phylogenetic tree. Indeed, several southern stuttering frog cathelicidins have the same signal and

cathelin region, but a different mature peptide. Other southern stuttering frog cathelicidins clustered

with cathelicidins from European and African frog species (Figure 2). This suggests these

cathelicidins may be the result of gene duplication events in a more distant common ancestor.

However, due in part to the great variability in AMP sequences, many of the relationships identified

were not strongly supported, particularly for β-defensins, limiting the validity of insights drawn from

these trees. It also remains difficult to ascertain whether the species-specific clusters of southern

stuttering frog cathelicidins are truly unique to *M. australis* or the *Mixophyes* genus more broadly, as

there are currently no other characterised cathelicidins from *Mixophyes* frogs. Additionally, the

number of known cathelicidins and β-defensins across the Anuran order is limited, with only one

cathelicidin characterised in a European frog, and no known cathelicidins or β-defensins from South

American frog species. Evolutionary relationships across these geographical regions are likely not

captured. As more frog cathelicidins and β-defensins are characterised, future investigations may be

able to better identify the evolutionary patterns of AMP diversity across all frogs.

The characterised and predicted properties of the stuttering frog AMPs suggest that they may play

diverse immunological roles. AMPs are generally cationic and amphipathic due to their electrostatic

interactions with anionic glycolipids on prokaryotic membranes, which facilitate membrane

permeability and cell lysis (Hancock and Rozek 2002; Rivas et al. 2009). However, AMPs can also exhibit diverse activities beyond antimicrobial activity and may serve other immune functions. For example, an anionic cathelicidin from a salamander species (TK-CATH) had no tested antimicrobial activity, but instead inhibited pro-inflammatory cytokine gene expression when added to mammalian macrophage cell lines (Luo et al. 2021). Two of our AMPs were anionic (MA-CATH8 and MA-BD2; Table S4), suggesting that they may have other immune functions. Future investigations will need to validate these *in silico* findings, such as by synthesising these AMPs and investigating their effects on immune gene expression in a range of cell lines.

While our bioinformatic characterisation of novel AMPs has demonstrated one application of the newly generated genomic and transcriptomic resources, there are numerous other potential applications. For instance, custom DNA metabarcoding markers generated from the mitochondrial genome can now be developed for the southern stuttering frog, contributing to applied conservation outcomes in this threatened species. Metabarcoding has been extensively used to characterise the biodiversity of different environments (Ruppert et al. 2019). Mitochondrial metagenomics (mtMG) has been previously used to distinguish between closely related species of nematodes (Gendron et al. 2023). As the southern stuttering frog is a recently defined species, closely related to the northern population of stuttering frogs (*M. balbus*) (Mahony et al. 2023), the mitochondrial genome generated in this study may be a useful monitoring tool, particularly in defining their range and overlap if applicable. Highly contiguous genomes and transcriptomes can also be used to characterise genomic regions that have high repeat content, such as the major histocompatibility complex (MHC) (Vekemans et al. 2021). In frogs, the upregulation of MHC class I and II genes in the Montane brown frog (*Rana ornativentris*) has a functional role in tadpole development (Lau et al. 2020). MHC heterozygosity is also a significant predictor of chytrid fungus resistance in the *Lithobates* genus (Savage and Zamudio 2011; Trujillo et al. 2021). Finally, these resources may be used comparatively with other genomes to investigate conserved and specialised traits across taxa. For example, emerging consortia like the Zoonomia Project have made significant progress in advancing our understanding of mammalian adaptations and evolutionary history (Christmas et al. 2023). Similar efforts in Anurans

would advance our understanding of a wealth of unique traits; some frog species can survive in

extreme temperatures and environments (Larson et al. 2014; van Beurden 1980), produce a myriad of

toxins (Raaymakers et al. 2017), and regenerate lost appendages as tadpoles (Aztekin et al. 2019). The

incorporation of the southern stuttering frog genome in future studies into amphibians will facilitate a

better representation of evolutionarily unique Australian biodiversity in these investigations. In short,

generating high-quality multi-omics resources facilitates a plethora of investigations into the southern

stuttering frog and amphibians at large.

METHODS

Sampling, Extractions and Sequencing

A wild caught, adult, male stuttering frog was medically euthanised in September 2021 (32°59'52.4"S

151°24'27.3"E). Heart and kidney tissue was flash frozen in liquid nitrogen for DNA extraction.

Gonads, brain, dorsal skin, ventral skin, liver, and spleen tissue were stored in RNAlater at -80°C

until RNA extraction. Lethal sampling was conducted under the University of Newcastle Animal Care

and Ethics Committee (ACEC Number A-2013-339) and NSW scientific licence (SL190).

DNA was extracted from heart and kidney tissue using a Nanobind Tissue Big DNA Kit

(Circulomics). Total extracted DNA was verified to be >20μg through Qubit fluorometric

quantification (ThermoFisher Scientific). DNA was pooled and sequenced at the Australian Genome

Research Facility (Brisbane, Queensland, Australia) using a SMRTbell® prep kit 3.0 (PacBio), and

circular consensus sequencing (CCS) was performed using three SMRT cells on a PacBio Sequel II

system. For Hi-C sequencing, heart and kidney tissue were washed twice for 5 minutes with 1×PBS

using a rotator wheel at room temperature. Tissues were sequenced at the Biomolecular Resource

Facility (Canberra, ACT, Australia), using the Arima Hi-C kit and sequenced as 150-bp paired-end

(PE) reads on an Illumina NovaSeq 6000.

RNA was extracted from the six tissues using a Qiagen RNeasy Mini Kit. Concentrations of each

sample were confirmed to be ≥25ng/μl using a Nanodrop spectrophotometer (ThermoFisher

Scientific) and the RNA integrity number (RIN) measured using the standard Agilent RNA 6000

Nano Kit Protocol and BioAnalyzer (Agilent Technologies). Extractions <7 RIN were not sequenced.

Extracted RNA was prepared at the Ramaciotti Centre for Genomics (Sydney, NSW, Australia), using

the Illumina Stranded mRNA Prep Protocol and sequenced as 100-bp PE reads on an Illumina

NovaSeq 6000 S1 flowcell.

De novo Genome Assembly

Over 15 million raw HiFi reads were generated from three SMRT cells. To prevent low quality reads

introducing errors to the assembly, reads with Phred (Q) quality score <20 were filtered out using

bamtools v2.4.1 (Barnett et al. 2011). Reads containing adapter sequence were removed by

HiFiAdapterFilt v2.0.0 (Sim et al. 2022) with default parameters. Further details on the computational

requirements and estimated run times for all bioinformatic analyses are provided in Table S6. Hifiasm

(Cheng et al. 2021) assembled the remaining reads, alongside paired Hi-C reads, into contigs. To

merge the contigs into a scaffolded assembly, we first mapped the Hi-C reads to the unscaffolded

genome following the Arima Hi-C mapping pipeline (A160156 v02;

https://github.com/ArimaGenomics/mapping_pipeline). YaHS (Zhou et al. 2022) was used to merge

contigs containing complementary pairs of reads, and the contact map visualised with Juicebox

(Durand et al. 2016). One misassembly in the first scaffold was manually corrected. We used

MitoHiFi v3.2 to identify and annotate the mitochondrial genome using a closely-related mitogenome

as input (*Lechriodus melanopyga*; NC_019999.1) (Allio et al. 2020; Uliano-Silva et al. 2023).

Genome statistics (e.g., N50 and L50 values), were calculated with bbmap v38.86

(https://sourceforge.net/projects/bbmap/). We identified regions matching canonical telomere hexamer

repeats (TTAGGG/CCCTAA) using FindTelomeres

(https://github.com/JanaSperschneider/FindTelomeres). Benchmarking Universal Single-Copy

Orthologs (BUSCO) v5.3.2 (Simão et al. 2015) analysis was performed on Galaxy Australia (Afgan et

al. 2018), using the 'genome' mode, applying the 'augustus' gene-finding setting, and with the

vertebrata_odb10 lineage. To compare the gene completeness of the stuttering frog with other

genomes, BUSCO analysis was also performed with the same settings on two model frog genomes,

the African clawed frog (*Xenopus laevis*; GCA_017654675.1) and the western clawed frog (*Xenopus*

tropicalis; GCA_000004195.4), as well as the three publicly available scaffolded Australian frog

genomes, the southern banjo frog (*Limnodynastes dumerilii*; GCA_011038615.1)*,* ornate burrowing

frog (*Platyplectrum ornatum*; GCA_016617825.1), and corroboree frog (*Pseudophryne corroboree*;

GCA_028390025.1). An alternative assessment of genome completeness, inclusive of non-coding and

repetitive regions, was performed for the stuttering frog and *P. ornatum* genomes using Merqury v1.3

(Rhie et al. 2020). RepeatModeler v2.0.1 (Flynn et al. 2020) was used to generate a *de novo* database

of the repetitive regions in the stuttering frog genome. We characterised repeats and masked the

genome with RepeatMasker v4.0.6 (Smit et al. 2013-2015). The repeat-masked genome was indexed

with hisat2 v2.1.0 (Kim et al. 2015).

Reference-aligned global transcriptome assembly

Over 900 million PE reads were generated across the six tissue transcriptomes. Low quality sequence

calls, adapter and primer sequences were trimmed using Trimmomatic v0.39 (Bolger et al. 2014) with

ILLUMINACLIP:TruSeq3-PE.fa:2:30:10, SLIDINGWINDOW:4:5, LEADING:5, TRAILING:5, and

MINLEN:25 settings. The reads from each tissue sample were aligned to the genome with hisat2

v2.1.0 (Kim et al. 2015). StringTie v2.1.6 (Pertea et al. 2015) was used to merge aligned reads into

tissue-specific transcriptomes. We used Transcriptome Annotation by Modular Algorithms (TAMA)

v1.0 (Kuo et al. 2020) to combine the tissue-specific transcriptomes into a global transcriptome with

adjustments to minimise duplicate transcripts. Briefly, the '-d merge_dup' flag was applied to merge

identical transcripts, and the '-z 500' flag was applied to facilitate transcripts with variable 3' ends

(differences of up to 500bp) to be merged. Transcripts with weak evidence were removed, including

transcripts that were found in only one tissue and were lowly expressed (fragments per kilobase of

transcript per million fragments mapped [FPKM] < 0.1). CPC2 v2019-11-19 (Kang et al. 2017) was

used to predict coding regions; non-coding regions were removed. Open reading frames were

predicted by TransDecoder v2.0.1 (Haas et al. 2013). BUSCO v5.3.2 (Simão et al. 2015) analysis was

performed using 'transcriptome' mode for both tissue-specific transcriptomes and the global

transcriptome, as well as the *X. tropicalis* transcriptome for comparison.

Genome Annotation

Genome Annotation

The genome was annotated with FGENESH++ v7.2.2 (Solovyev et al. 2006) informed by the global

transcriptome. Non-mammalian settings were applied throughout the pipeline, and parameters were

optimised for Anuran gene discovery by providing the *Xenopus* gene-finding matrix (Softberry).

BUSCO v5.3.2 (Simão et al. 2015) analysis was performed on the annotation using 'protein' mode, as

well as for the *X. laevis, X. tropicalis* and *L. dumerilii* annotations. The number of genes, exons and

introns from the stuttering frog annotated assembly was calculated using the 'genestats' script

(https://github.com/darencard/GenomeAnnotation/blob/master/genestats).

AMP characterisation

AMP characterisation

To ensure a comprehensive search of genomic regions that could encode cathelicidins or β-defensins,

the annotated genome and global transcriptome were queried using Basic Local Alignment Search

Tool (BLAST) v2.2.30+ (Altschul et al. 1990; Altschul et al. 1997) and HMMER v3.3 (Eddy 2011).

A dual program approach has previously been applied in other bioinformatic searches for cathelicidins

and β-defensins (Helbing et al. 2019; Schutte et al. 2002; Whittington et al. 2008). Further

explanation of the approaches used is provided in the Supplementary Extended Methods.

AMP phylogeny

AMP phylogeny

An unrooted phylogenetic tree was generated for the cathelicidin and β-defensin gene families. We

first aligned amino acid sequences for each gene family through ClustalW alignments. Both

alignments included the full prepropeptide sequences from the stuttering frog and other available

frogs (Table S5). The cathelicidin alignment also included four fish cathelicidins as an outgroup

(Table S5). The best-fitting substitution models for each alignment were selected using the

ModelFinder option in IQ-TREE v2.2.0 (Minh et al. 2020) according to the Bayesian information

criterion (Kalyaanamoorthy et al. 2017). For the cathelicidin alignment, the Jones Taylor Thornton

(JTT) substitution model (Jones et al. 1992) was optimal, incorporating Invariant Sites (+I) and four

components of gamma rate heterogeneity (+G4). For the β-defensin alignment, the Dayhoff model

was optimal (Dayhoff et al. 1978). Maximum likelihood analysis was conducted in IQ-TREE v2.2.0

(Minh et al. 2020) with node support estimated by ultrafast bootstrap approximation with 1000

replicates (Hoang et al. 2017). The results were visualised and annotated using MEGA11 (Tamura et

al. 2021).

Characterisation and Prediction of AMP Structure, Properties and Expression

Cathelicidins are made up of a signal peptide, a conserved cathelin pro-region, as well as the mature,

bioactive peptide (Chen et al. 2021; Tomasinsig and Zanetti 2005). To predict the signal peptide from

the full prepropeptide sequence, the SignalP v6.0 webserver was used (Teufel et al. 2022) with the

'Eukarya' organism setting and the 'Slow' model mode. While the enzyme used to cleave the mature

peptide from the cathelin pro-region is not known for amphibians, there is experimental evidence to

support proprotein convertases or trypsin-like proteases that cleave at dibasic residues like lysine (K)

and arginine (R) (Mu et al. 2017; Shi et al. 2020; Yu et al. 2013). Therefore, a two-tiered approach

predicted the mature peptide of the novel cathelicidins. Proprotein convertase cleavage sites were first

predicted from the last exon of the cathelicidins using ProP v1.0 (Duckert et al. 2004). If no cleavage

was identified, trypsin cleavage sites were predicted using ExPASy's peptide cutter tool (Gasteiger et

al. 2003). For β-defensins, which generally consist of a signal peptide and a mature, bioactive peptide

(Huttner and Bevins 1999; Semple and Dorin 2012; Tang and Selsted 1993), the signal peptide was

predicted using SignalP, and the remaining peptide was annotated as the mature peptide.

The molecular weight and charge at pH 7 for each putative stuttering frog cathelicidin and β-defensin

was calculated using Protein Calculator v3.4 (https://protcalc.sourceforge.net/). The percentage of

hydrophobic residues in the AMPs was calculated on Peptide v2.0 (https://www.peptide2.com/) using

the 'Peptide Hydrophobicity/Hydrophilicity Analysis' tool. The amphipathicity of the AMPs was

determined by generating Kyte and Doolittle hydropathicity plots on ExPASy using Protscale

(Gasteiger et al. 2003), with a window size of 5. These plots were inspected for the presence of

'peaks' and 'troughs', which indicate sections in the AMP of high and low hydropathicity (Peel et al.

2017). For the expression analysis, the Transcripts Per Million (TPM) values for each AMP was

generated from the tissue-specific transcriptomes using StringTie v2.1.6 (Pertea et al. 2015).

DATA ACCESS

The genome assembly, mitochondrial genome, and raw transcriptome sequencing data generated in this study have been submitted to the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJNA991157. Raw genome sequencing data is publicly available through the Bioplatforms Australia Threatened Species Initiative (https://data.bioplatforms.com/organization/threatened-species). The global transcriptome and annotation generated in this study are available on Amazon Web Services Australasian Genomes Open Data Store (https://awgg-lab.github.io/australasiangenomes/genomes.html).

COMPETING INTEREST STATEMENT

The authors have no competing interests to declare.

ACKNOWLEDGMENTS

We acknowledge the traditional custodians of the land where the specimen was collected, the Awabakal people, and pay respects to their elders past and present, and their ongoing connection to country. Thank you to M. Mahony, University of Newcastle, who collected the specimen. Thanks also to Elspeth McLennan for assistance with the DNA and RNA extractions. Computational resources were provided by Amazon Web Services and RONIN; the Australian FGENESH++ Service provided by the Australian Biocommons and the Pawsey Supercomputing Research Centre with funding from the Australian Government and the Government of Western Australia; Galaxy Australia, a service provided by the Australian Biocommons and its partners; and the University of Sydney's High Performance Computing facility Artemis provided by the Sydney Informatics Hub. The authors wish to acknowledge the use of the services and facilities of the Australian Genome Research Facility; the Ramaciotti Centre for Genomics, UNSW; and the Biomolecular Resource Facility, Canberra. Funding was provided by the Threatened Species Initiative through a grant from the Australian Government Bushfire Recovery Scheme (ERF-WRR2-020), and the Australian Research Council Centre of Excellence for Innovations in Peptide and Protein Science (CE200100012).

AUTHOR CONTRIBUTIONS

465   The project was conceived, and funding sought, by CJH and KB. ST undertook the genome and

466   transcriptome assembly with assistance from KAF. ST characterised the AMPs with assistance from

467   KAF and EP. ST drafted the manuscript with assistance from KAF. All authors provided review and

468   feedback.

REFERENCES

Afgan E, Baker D, Batut B, van den Beek M, Bouvier D, Čech M, Chilton J, Clements D, Coraor N, Grüning BA, et al. 2018. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res* **46**: 537-544. doi:10.1093/nar/gky379

Allio R, Schomaker-Bastos A, Romiguier J, Prosdocimi F, Nabholz B, Delsuc F. 2020. MitoFinder: Efficient automated large-scale extraction of mitogenomic data in target enrichment phylogenomics. *Mol Ecol Resour* **20**: 892-905. doi:10.1111/1755-0998.13160

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic Local Alignment Search Tool. *J Mol Biol* **215**: 403-410. doi:10.1006/jmbi.1990.9999

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389-3402. doi:10.1093/nar/25.17.3389

AmphibiaWeb. 2022. *Amphibian Species By the Numbers*. University of California, California. https://amphibiaweb.org/amphibian/speciesnums.html

Aztekin C, Hiscock TW, Marioni JC, Gurdon JB, Simons BD, Jullien J. 2019. Identification of a regeneration-organizing cell in the *Xenopus* tail. *Science* **364**: 653-658. doi:10.1126/science.aav9996

Barker J, Grigg G, Tyler MJ. 1995. *A Field Guide to Australian frogs*. Surrey Beatty and Sons, New South Wales.

Barnett DW, Garrison EK, Quinlan AR, Strömberg MP, Marth GT. 2011. BamTools: a C++ API and toolkit for analyzing and managing BAM files. *Bioinformatics* **27**: 1691-1692. doi:10.1093/bioinformatics/btr174

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114-2120. doi:10.1093/bioinformatics/btu170

Brennan IG, Lemmon AR, Lemmon EM, Hoskin CJ, Donnellan SC, Keogh JS. 2023. Populating a Continent: Phylogenomics Reveal the Timing of Australian Frog Diversification. *Syst Biol*. doi:10.1093/sysbio/syad048

Chen CH, Lu TK. 2020. Development and Challenges of Antimicrobial Peptides for Therapeutic Applications. *Antibiotics (Basel)* **9**. doi:10.3390/antibiotics9010024

Chen J, Lin YF, Chen JH, Chen X, Lin ZH. 2021. Molecular characterization of cathelicidin in tiger frog (Hoplobatrachus rugulosus): Antimicrobial activity and immunomodulatory activity. *Comp Biochem Physiol C Toxicol Pharmacol* **247**: 109072. doi:10.1016/j.cbpc.2021.109072

Cheng H, Concepcion GT, Feng X, Zhang H, Li H. 2021. Haplotype-resolved de novo assembly using phased assembly graphs with hifiasm. *Nat Methods* **18**: 170-175. doi:10.1038/s41592-020-01056-5

Christmas MJ, Kaplow IM, Genereux DP, Dong MX, Hughes GM, Li X, Sullivan PF, Hindle AG, Andrews G, Armstrong JC, et al. 2023. Evolutionary constraint and innovation across hundreds of placental mammals. *Science* **380**: eabn3943. doi:10.1126/science.abn3943

Cogger HG. 2000. *Reptiles and Amphibians of Australia* (6th ed.). Reed New Holland, New South Wales.

Dalla Valle L, Benato F, Maistro S, Quinzani S, Alibardi L. 2012. Bioinformatic and molecular characterization of beta-defensins-like peptides isolated from the green lizard Anolis carolinensis. *Dev Comp Immunol* **36**: 222-229. doi:10.1016/j.dci.2011.05.004

Dayhoff M, Schwartz R, Orcutt B. 1978. A model of evolutionary change in proteins. In *Atlas of Protein Sequence and Structure*, pp. 345-352. National Biomedical Research Foundation, Washington DC.

Donnellan SC, Mahony MJ, Davies M. 1990. A New Species of Mixophyes (Anura: Leptodactylidae) and First Record of the Genus in New Guinea. *Herpetologica* **46**: 266-274. https://www.jstor.org/stable/3892969

Duckert P, Brunak S, Blom N. 2004. Prediction of proprotein convertase cleavage sites. *Protein Eng*
Des Sel **17**: 107-112. doi:10.1093/protein/gzh013
Durand NC, Robinson JT, Shamim MS, Machol I, Mesirov JP, Lander ES, Aiden EL. 2016. Juicebox
Provides a Visualization System for Hi-C Contact Maps with Unlimited Zoom. *Cell Syst* **3**: 99-
101. doi:10.1016/j.cels.2015.07.012
Eddy SR. 2011. Accelerated Profile HMM Searches. *PLoS Comput Biol* **7**: e1002195.
doi:10.1371/journal.pcbi.1002195
Farquharson K, McLennan E, Belov K, Hogg C. 2023. The genome sequence of the critically
endangered Kroombit tinkerfrog (*Taudactylus pleione*). *F1000Research* **12**.
doi:10.12688/f1000research.138571.1
Flynn JM, Hubley R, Goubert C, Rosen J, Clark AG, Feschotte C, Smit AF. 2020. RepeatModeler2 for
automated genomic discovery of transposable element families. *Proc Natl Acad Sci USA* **117**:
9451-9457. doi:10.1073/pnas.1921046117
Formenti G, Theissinger K, Fernandes C, Bista I, Bombarely A, Bleidorn C, Ciofi C, Crottini A, Godoy J,
Höglund J, et al. 2022. The era of reference genomes in conservation genomics. *Trends Ecol*
Evol **37**. doi:10.1016/j.tree.2021.11.008
Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: The proteomics
server for in-depth protein knowledge and analysis. *Nucleic Acids Res* **31**: 3784-3788.
doi:10.1093/nar/gkg563
Gendron EM, Sevigny JL, Byiringiro I, Thomas WK, Powers TO, Porazinska DL. 2023. Nematode
mitochondrial metagenomics: A new tool for biodiversity analysis. *Mol Ecol Resour* **23**: 975-
989. doi:10.1111/1755-0998.13761
Grant JR, Enns E, Marinier E, Mandal A, Herman EK, Chen CY, Graham M, Van Domselaar G, Stothard
P. 2023. Proksee: in-depth characterization and visualization of bacterial genomes. *Nucleic*
Acids Res **51**: 484-492. doi:10.1093/nar/gkad326
Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B,
Lieber M, et al. 2013. De novo transcript sequence reconstruction from RNA-seq using the
Trinity platform for reference generation and analysis. *Nat Protoc* **8**: 1494-1512.
doi:10.1038/nprot.2013.084
Hancock REW, Rozek A. 2002. Role of membranes in the activities of antimicrobial cationic peptides.
FEMS Microbiol Lett **206**: 143-149. doi:10.1111/j.1574-6968.2002.tb11000.x
Hanson MA, Lemaitre B, Unckless RL. 2019. Dynamic Evolution of Antimicrobial Peptides
Underscores Trade-Offs Between Immunity and Ecological Fitness. *Front Immunol* **10**.
doi:10.3389/fimmu.2019.02620
Hao X, Yang H, Wei L, Yang S, Zhu W, Ma D, Yu H, Lai R. 2012. Amphibian cathelicidin fills the
evolutionary gap of cathelicidin in vertebrate. *Amino Acids* **43**: 677-685.
doi:10.1007/s00726-011-1116-7
He X, Yang Y, Mu L, Zhou Y, Chen Y, Wu J, Wang Y, Yang H, Li M, Xu W, et al. 2019. A Frog-Derived
Immunomodulatory Peptide Promotes Cutaneous Wound Healing by Regulating Cellular
Response. *Front Immunol* **10**: 2421. doi:10.3389/fimmu.2019.02421
Helbing CC, Hammond SA, Jackman SH, Houston S, Warren RL, Cameron CE, Birol I. 2019.
Antimicrobial peptides from *Rana* [*Lithobates*] *catesbeiana*: Gene structure and
bioinformatic identification of novel forms from tadpoles. *Sci Rep* **9**: 1529.
doi:10.1038/s41598-018-38442-1
Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. 2017. UFBoot2: Improving the Ultrafast
Bootstrap Approximation. *Mol Biol and Evol* **35**: 518-522. doi:10.1093/molbev/msx281
Hollanders M, Grogan LF, Nock CJ, McCallum HI, Newell DA. 2023. Recovered frog populations
coexist with endemic *Batrachochytrium dendrobatidis* despite load-dependent mortality.
Ecol Appl **33**: e2724. doi:10.1002/eap.2724
Horgan R, Kenny L. 2011. 'Omic' technologies: Genomics, transcriptomics, proteomics and
metabolomics. *The Obstetrician & Gynaecologist* **13**. doi:10.1576/toag.13.3.189.27672

Hotaling S, Kelley J, Frandsen P. 2021. Toward a genome sequence for every animal: Where are we now? *Proc Natl Acad Sci USA* **118**: e2109019118. doi:10.1073/pnas.2109019118

Huan YC, Kong Q, Mou HJ, Yi HX. 2020. Antimicrobial Peptides: Classification, Design, Application and Research Progress in Multiple Fields. *Front Microbiol* **11**. doi:10.3389/fmicb.2020.582779

Huttner KM, Bevins CL. 1999. Antimicrobial peptides as mediators of epithelial host defense. *Pediatr Res* **45**: 785-794. doi:10.1203/00006450-199906000-00001

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* **8**: 275-282. doi:10.1093/bioinformatics/8.3.275

Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods* **14**: 587-589. doi:10.1038/nmeth.4285

Kang Y-J, Yang D-C, Kong L, Hou M, Meng Y-Q, Wei L, Gao G. 2017. CPC2: a fast and accurate coding potential calculator based on sequence intrinsic features. *Nucleic Acids Res* **45**: W12-W16. doi:10.1093/nar/gkx428

Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* **12**: 357-360. doi:10.1038/nmeth.3317

Kosciuczuk EM, Lisowski P, Jarczak J, Strzalkowska N, Jozwik A, Horbanczuk J, Krzyzewski J, Zwierzchowski L, Bagnicka E. 2012. Cathelicidins: family of antimicrobial peptides. A review. *Mol Biol Rep* **39**: 10957-10970. doi:10.1007/s11033-012-1997-x

Kuo RI, Cheng Y, Zhang R, Brown JWS, Smith J, Archibald AL, Burt DW. 2020. Illuminating the dark side of the human transcriptome with long read transcript sequencing. *BMC Genomics* **21**: 751. doi:10.1186/s12864-020-07123-7

Ladram A, Nicolas P. 2016. Antimicrobial peptides from frog skin: biodiversity and therapeutic promises. *Front Biosci-Landmrk* **21**: 1341-1371. doi:10.2741/4461

Lamichhaney S, Catullo R, Keogh JS, Clulow S, Edwards SV, Ezaz T. 2021. A bird-like genome from a frog: Mechanisms of genome size reduction in the ornate burrowing frog, *Platyplectrum ornatum*. *Proc Natl Acad Sci USA* **118**. doi:10.1073/pnas.2011649118

Larson DJ, Middle L, Vu H, Zhang W, Serianni AS, Duman J, Barnes BM. 2014. Wood frog adaptations to overwintering in Alaska: new limits to freezing tolerance. *J Exp Biol* **217**: 2193-2200. doi:10.1242/jeb.101931

Lau Q, Igawa T, Komaki S, Satta Y. 2020. Expression Changes of MHC and Other Immune Genes in Frog Skin during Ontogeny. *Animals (Basel)* **10**. doi:10.3390/ani10010091

Lewin HA, Richards S, Lieberman Aiden E, Allende ML, Archibald JM, Bálint M, Barker KB, Baumgartner B, Belov K, Bertorelle G, et al. 2022. The Earth BioGenome Project 2020: Starting the clock. *Proc Natl Acad Sci USA* **119**. doi:10.1073/pnas.2115635118

Lewin HA, Robinson GE, Kress WJ, Baker WJ, Coddington J, Crandall KA, Durbin R, Edwards SV, Forest F, Gilbert MTP, et al. 2018. Earth BioGenome Project: Sequencing life for the future of life. *Proc Natl Acad Sci USA* **115**: 4325-4333. doi:10.1073/pnas.1720115115

Li Q, Guo Q, Zhou Y, Tan H, Bertozzi T, Zhu Y, Li J, Donnellan S, Zhang G. 2020. A draft genome assembly of the eastern banjo frog *Limnodynastes dumerilii dumerilii* (Anura: Limnodynastidae). *Gigabyte* **2020**: 1-13. doi:10.46471/gigabyte.2

Liedtke HC, Gower DJ, Wilkinson M, Gomez-Mestre I. 2018. Macroevolutionary shift in the size of amphibian genomes and the role of life history and climate. *Nat Ecol Evol* **2**: 1792-1799. doi:10.1038/s41559-018-0674-4

Ling G, Gao J, Zhang S, Xie Z, Wei L, Yu H, Wang Y. 2014. Cathelicidins from the Bullfrog *Rana catesbeiana* Provides Novel Template for Peptide Antibiotic Design. *PLoS One* **9**: e93216. doi:10.1371/journal.pone.0093216

Luo XJ, Ouyang JH, Wang Y, Zhang MH, Fu L, Xiao N, Gao LH, Zhang P, Zhou J, Wang YP. 2021. A novel anionic cathelicidin lacking direct antimicrobial activity but with potent anti-inflammatory and wound healing activities from the salamander *Tylototriton kweichowensis*. *Biochimie* **191**: 37-50. doi:10.1016/j.biochi.2021.08.007

620 Mahony M, Bertozzi T, Guzinski J, Hines H, Donnellan S. 2023. A new species of barred frog,
621    Mixophyes (Anura: Myobatrachidae) from south-eastern Australia identified by molecular
622    genetic analyses. *Zootaxa* **5297**: 301-336. doi:10.11646/zootaxa.5297.3.1
623 Mercer DK, Robertson JC, Miller L, Stewart CS, O'Neil DA. 2020. NP213 (Novexatin®): A unique
624    therapy candidate for onychomycosis with a differentiated safety and efficacy profile. *Med*
625    *Mycol* **58**: 1064-1072. doi:10.1093/mmy/myaa015
626 Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R. 2020.
627    IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic
628    Era. *Mol Biol Evol* **37**: 1530-1534. doi:10.1093/molbev/msaa015
629 Mu L, Zhou L, Yang J, Zhuang L, Tang J, Liu T, Wu J, Yang H. 2017. The first identified cathelicidin from
630    tree frogs possesses anti-inflammatory and partial LPS neutralization activities. *Amino Acids*
631    **49**: 1571-1585. doi:10.1007/s00726-017-2449-7
632 Murray BR, Hose GC. 2005. Life-history and ecological correlates of decline and extinction in the
633    endemic Australian frog fauna. *Austral Ecol* **30**: 564-571. doi:DOI 10.1111/j.1442-
634    9993.2005.01471.x
635 Novikova PY, Brennan IG, Booker W, Mahony M, Doughty P, Lemmon AR, Moriarty Lemmon E,
636    Roberts JD, Yant L, Van de Peer Y, et al. 2020. Polyploidy breaks speciation barriers in
637    Australian burrowing frogs Neobatrachus. *PLoS Genet* **16**: e1008769.
638    doi:10.1371/journal.pgen.1008769
639 Novković M, Simunić J, Bojović V, Tossi A, Juretić D. 2012. DADP: the database of anuran defense
640    peptides. *Bioinformatics* **28**: 1406-1407. doi:10.1093/bioinformatics/bts141
641 Paez S, Kraus RHS, Shapiro B, Gilbert MTP, Jarvis ED, Al-Ajli FO, Ceballos G, Crawford AJ, Fedrigo O,
642    Johnson RN, et al. 2022. Reference genomes for conservation. *Science* **377**: 364-366.
643    doi:10.1126/science.abm8127
644 Peel E, Cheng Y, Djordjevic JT, Fox S, Sorrell TC, Belov K. 2016. Cathelicidins in the Tasmanian devil
645    (*Sarcophilus harrisii*). *Sci Rep* **6**: 35019. doi:10.1038/srep35019
646 Peel E, Cheng Y, Djordjevic JT, Kuhn M, Sorrell T, Belov K. 2017. Marsupial and monotreme
647    cathelicidins display antimicrobial activity, including against methicillin-resistant
648    *Staphylococcus aureus*. *Microbiology (Reading)* **163**: 1457-1465. doi:10.1099/mic.0.000536
649 Pérez de la Lastra JM, Asensio-Calavia P, González-Acosta S, Baca-González V, Morales-delaNuez A.
650    2021. Bioinformatic Analysis of Genome-Predicted Bat Cathelicidins. *Molecules* **26**.
651    doi:10.3390/molecules26061811
652 Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015. StringTie enables
653    improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* **33**: 290-
654    295. doi:10.1038/nbt.3122
655 Raaymakers C, Verbrugghe E, Hernot S, Hellebuyck T, Betti C, Peleman C, Claeys M, Bert W, Caveliers
656    V, Ballet S, et al. 2017. Antimicrobial peptides in frog poisons constitute a molecular toxin
657    delivery system against predators. *Nat Commun* **8**: 1495. doi:10.1038/s41467-017-01710-1
658 Rhie A, McCarthy SA, Fedrigo O, Damas J, Formenti G, Koren S, Uliano-Silva M, Chow W,
659    Fungtammasan A, Kim J, et al. 2021. Towards complete and error-free genome assemblies of
660    all vertebrate species. *Nature* **592**: 737-746. doi:10.1038/s41586-021-03451-0
661 Rhie A, Walenz BP, Koren S, Phillippy AM. 2020. Merqury: reference-free quality, completeness, and
662    phasing assessment for genome assemblies. *Genome Biol* **21**: 245. doi:10.1186/s13059-020-
663    02134-9
664 Ridyard KE, Overhage J. 2021. The Potential of Human Peptide LL-37 as an Antimicrobial and Anti-
665    Biofilm Agent. *Antibiotics (Basel)* **10**. doi:10.3390/antibiotics10060650
666 Rivas L, Luque-Ortega JR, Andreu D. 2009. Amphibian antimicrobial peptides and Protozoa: Lessons
667    from parasites. *Biochim Biophys Acta* **1788**: 1570-1581. doi:10.1016/j.bbamem.2008.11.002
668 Ruppert KM, Kline RJ, Rahman MS. 2019. Past, present, and future perspectives of environmental
669    DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications

of global eDNA. *Global Ecology and Conservation* **17**: e00547.
doi:10.1016/j.gecco.2019.e00547
Savage A, Zamudio K. 2011. MHC genotypes associate with resistance to a frog-killing fungus. *Proc*
Natl Acad Sci USA **108**: 16705-16710. doi:10.1073/pnas.1106893108
Sayers EW, Bolton EE, Brister JR, Canese K, Chan J, Comeau DC, Connor R, Funk K, Kelly C, Kim S, et
al. 2022. Database resources of the national center for biotechnology information. *Nucleic*
Acids Res **50**: 20-26. doi:10.1093/nar/gkab1112
Schmid M, Haaf T, Steinlein C, Nanda I, Mahony M. 2002. Chromosome banding in Amphibia. XXV.
Karyotype evolution and heterochromatin characterization in Australian *Mixophyes* (Anura,
Myobatrachidae). *Cytogenet Genome Res* **97**: 239-253. doi:10.1159/000066614
Schutte BC, Mitros JP, Bartlett JA, Walters JD, Jia HP, Welsh MJ, Casavant TL, McCray PB, Jr. 2002.
Discovery of five conserved beta -defensin gene clusters using a computational search
strategy. *Proc Natl Acad Sci USA* **99**: 2129-2133. doi:10.1073/pnas.042692699
Seidl F, Levis NA, Schell R, Pfennig DW, Pfennig KS, Ehrenreich IM. 2019. Genome of *Spea*
multiplicata, a Rapidly Developing, Phenotypically Plastic, and Desert-Adapted Spadefoot
Toad. *G3 Genes|Genomes|Genetics* **9**: 3909-3919. doi:10.1534/g3.119.400705
Semple F, Dorin JR. 2012. β-Defensins: Multifunctional Modulators of Infection, Inflammation and
More? *J Innate Immun* **4**: 337-348. doi:10.1159/000336619
Shen W, Chen Y, Yao H, Du C, Luan N, Yan X. 2016. A novel defensin-like antimicrobial peptide from
the skin secretions of the tree frog, *Theloderma kwangsiensis*. *Gene* **576**: 136-140.
doi:10.1016/j.gene.2015.09.086
Shi Y, Li C, Wang M, Chen Z, Luo Y, Xia XS, Song Y, Sun Y, Zhang AM. 2020. Cathelicidin-DM is an
Antimicrobial Peptide from Duttaphrynus melanostictus and Has Wound-Healing
Therapeutic Potential. *ACS Omega* **5**: 9301-9310. doi:10.1021/acsomega.0c00189
Sim SB, Corpuz RL, Simmonds TJ, Geib SM. 2022. HiFiAdapterFilt, a memory efficient read processing
pipeline, prevents occurrence of adapter sequence in PacBio HiFi reads and their negative
impacts on genome assembly. *BMC Genomics* **23**: 157. doi:10.1186/s12864-022-08375-1
Simão FA, Waterhouse RM, Ioannidis P, Kriventseva EV, Zdobnov EM. 2015. BUSCO: assessing
genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics*
31: 3210-3212. doi:10.1093/bioinformatics/btv351
Smit A, Hubley R, Green P. (2013-2015). *RepeatMasker Open-4.0*. http://www.repeatmasker.org
Smith JJ, Timoshevskaya N, Timoshevskiy VA, Keinath MC, Hardy D, Voss SR. 2019. A chromosome-
scale assembly of the axolotl genome. *Genome Res* **29**: 317-324. doi:10.1101/gr.241901.118
Solovyev V, Kosarev P, Seledsov I, Vorobyev D. 2006. Automatic annotation of eukaryotic genes,
pseudogenes and promoters. *Genome Biol* **7**: S10. doi:10.1186/gb-2006-7-s1-s10
Tamura K, Stecher G, Kumar S. 2021. MEGA11: Molecular Evolutionary Genetics Analysis Version 11.
Mol Biol Evol **38**: 3022-3027. doi:10.1093/molbev/msab120
Tang YQ, Selsted ME. 1993. Characterization of the disulfide motif in BNBD-12, an antimicrobial
beta-defensin peptide from bovine neutrophils. *J Biol Chem* **268**: 6649-6653.
doi:10.1016/S0021-9258(18)53299-3
Teufel F, Almagro Armenteros JJ, Johansen AR, Gíslason MH, Pihl SI, Tsirigos KD, Winther O, Brunak
S, von Heijne G, Nielsen H. 2022. SignalP 6.0 predicts all five types of signal peptides using
protein language models. *Nat Biotechnol* **40**: 1023-1025. doi:10.1038/s41587-021-01156-3
Tomasinsig L, Zanetti M. 2005. The cathelicidins--structure, function and evolution. *Curr Protein Pept*
Sci **6**: 23-34. doi:10.2174/1389203053027520
Trujillo AL, Hoffman EA, Becker CG, Savage AE. 2021. Spatiotemporal adaptive evolution of an MHC
immune gene in a frog-fungus disease system. *Heredity* **126**: 640-655. doi:10.1038/s41437-
020-00402-9
Tu J, Li D, Li Q, Zhang L, Zhu Q, Gaur U, Fan X, Xu H, Yao Y, Zhao X, et al. 2015. Molecular Evolutionary
Analysis of β-Defensin Peptides in Vertebrates. *Evol Bioinform Online* **11**: 105-114.
doi:10.4137/ebo.S25580

Uliano-Silva M, Ferreira J, Krasheninnikova K, Formenti G, Abueg L, Torrance J, Myers EW, Durbin R,
Blaxter M, McCarthy SA. 2023. MitoHiFi: a python pipeline for mitochondrial genome
assembly from PacBio high fidelity reads. *BMC Bioinformatics* **24**: 288. doi:10.1186/s12859-
023-05385-y
van Beurden EK. 1980. Energy Metabolism of Dormant Australian Water-Holding Frogs (*Cyclorana
platycephalus*). *Copeia* **1980**: 787-799. doi:10.2307/1444458
Varga JFA, Bui-Marinos MP, Katzenback BA. 2018. Frog Skin Innate Immune Defences: Sensing and
Surviving Pathogens. *Front Immunol* **9**: 3128. doi:10.3389/fimmu.2018.03128
Vekemans X, Castric V, Hipperson H, Müller NA, Westerdahl H, Cronk Q. 2021. Whole-genome
sequencing and genome regions of special interest: Lessons from major histocompatibility
complex, sex determination, and plant self-incompatibility. *Mol Ecol* **30**: 6072-6086.
doi:10.1111/mec.16020
Wang G, Li X, Wang Z. 2016. APD3: the antimicrobial peptide database as a tool for research and
education. *Nucleic Acids Res* **44**: 1087-1093. doi:10.1093/nar/gkv1278
Wang M, Zhou Z, Li S, Zhu W, Hu X. 2021. Identification and Characterization of Antimicrobial
Peptides From Butterflies: An Integrated Bioinformatics and Experimental Study. *Front
Microbiol* **12**: 720381. doi:10.3389/fmicb.2021.720381
Wang Q, Xia R, Ji JJ, Zhu Q, Li XP, Ma Y, Xu YC. 2020. Diversity of Antimicrobial Peptides in Three
Partially Sympatric Frog Species in Northeast Asia and Implications for Evolution. *Genes
(Basel)* **11**. doi:10.3390/genes11020158
Wei L, Che H, Han Y, Lv J, Mu L, Lv L, Wu J, Yang H. 2015. The first anionic defensin from amphibians.
Amino Acids **47**: 1301-1308. doi:10.1007/s00726-015-1963-8
Whittington CM, Papenfuss AT, Bansal P, Torres AM, Wong ES, Deakin JE, Graves T, Alsop A,
Schatzkamer K, Kremitzki C, et al. 2008. Defensins and the convergent evolution of platypus
and reptile venom genes. *Genome Res* **18**: 986-994. doi:10.1101/gr.7149808
Wong AK, Sealfon RSG, Theesfeld CL, Troyanskaya OG. 2021. Decoding disease: from genomes to
networks to phenotypes. *Nat Rev Genet* **22**: 774-790. doi:10.1038/s41576-021-00389-x
Woodhams DC, Ardipradja K, Alford RA, Marantelli G, Reinert LK, Rollins-Smith LA. 2007. Resistance
to chytridiomycosis varies among amphibian species and is correlated with skin peptide
defenses. *Anim Conserv* **10**: 409-417. doi:10.1111/j.1469-1795.2007.00130.x
Yang Y, Wu J, Li Q, Wang J, Mu L, Hui L, Li M, Xu W, Yang H, Wei L. 2022. A non-bactericidal
cathelicidin provides prophylactic efficacy against bacterial infection by driving phagocyte
influx. *eLife* **11**: e72849. doi:10.7554/eLife.72849
Yoo WG, Lee S, Lee MR, Yun MR, Kwon T, Kim DW. 2015. Genome-wide identification of
antimicrobial peptides in the liver fluke, *Clonorchis sinensis*. *Bioinformation* **11**: 17-20.
doi:10.6026/97320630011017
Yu H, Cai S, Gao J, Zhang S, Lu Y, Qiao X, Yang H, Wang Y. 2013. Identification and polymorphism
discovery of the cathelicidins, Lf-CATHs in ranid amphibian (*Limnonectes fragilis*). *FEBS J* **280**:
6022-6032. doi:10.1111/febs.12521
Yu SS, Zhao ZH, Gong XF, Fan XL, Lin ZH, Chen J. 2022. Antimicrobial and immunomodulatory activity
of beta-defensin from the Chinese spiny frog (*Quasipaa spinosa*). *Dev Comp Immunol* **126**:
104264. doi:10.1016/j.dci.2021.104264
Zhou C, McCarthy SA, Durbin R. 2022. YaHS: yet another Hi-C scaffolding tool. *Bioinformatics* **39**.
doi:10.1093/bioinformatics/btac808
765