Whole Snake Genomes from Eighteen Families of Snakes (Serpentes: Caenophidia) and Their Applications to Systematics

Jackson R. Roberts[1,2,3*], Justin M. Bernstein[4,5,*], Christopher C. Austin[2,3], Taylor Hains[6,7], Joshua Mata[8], Michael Kieras[9], Stacy Pirro[9], Sara Ruane[7,8]

[1] Sternberg Museum of Natural History, Fort Hays State University, Hays, Kansas, 67601, United States

[2] Division of Herpetology, Museum of Natural Science, Louisiana State University, Baton Rouge, Louisiana, 70803, United States

[3] Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, 70803, United States

[4] Center for Genomics, University of Kansas, Lawrence, Kansas, 66045, United States

[5] Department of Biology, University of Texas at Arlington, Arlington, Texas, 76010, United States

[6] Committee on Evolutionary Biology, University of Chicago, Chicago, Illinois, 60637, United States

[7] Life Sciences Section, Negaunee Integrative Research Center, The Field Museum of Natural History, Chicago, Illinois, 60637, United States

[8] Amphibian and Reptile Collection, The Field Museum of Natural History, Chicago, Illinois 60605, United States

[9] Iridian Genomes, Inc., Bethesda, Maryland, 20817, United States

Corresponding author: Jackson R. Roberts; [*]Both authors (JRR and Justin Bernstein) contributed equally to this work and are submitting this manuscript as co- first authors.

Abstract

We present genome assemblies for 18 snake species representing 18 families (Serpentes: Caenophidia): *Acrochordus granulatus*, *Aparallactus werneri*, *Boaedon fuliginosus*, *Calamaria suluensis*, *Cerberus rynchops*, *Grayia smithii*, *Imantodes cenchoa*, *Mimophis mahfalensis*, *Oxyrhabdium leporinum*, *Pareas carinatus*, *Psammodynastes pulverulentus*, *Pseudoxenodon macrops*, *Pseudoxyrhopus heterurus*, *Sibynophis collaris*, *Stegonotus admiraltiensis*, *Toxicocalamus goodenoughensis*, *Trimeresurus albolabris*, and *Tropidonophis doriae*. From these new genome assemblies, we extracted thousands of loci commonly used in systematic and phylogenomic studies on snakes, including target-capture datasets composed of UCEs and AHEs, as well as traditional Sanger loci. Phylogenies inferred from the two target-capture loci datasets were identical with each other, and strongly congruent with previously published snake phylogenies. To show additional utility of these non-model genomes for investigative evolutionary research, we mined the genome assemblies of two New Guinea island endemics in our dataset (*Stegonotus admiraltiensis* and *Tropidonophis doriae*) for the *ATP1a3* gene, a thoroughly researched indicator of resistance to toad toxin ingestion by squamates. We find that both these snakes possess the genotype for toad toxin resistance despite their endemism to New Guinea, a region absent of any toads until the human-mediated introduction of Cane Toads in the 1930s. These species possess identical substitutions that suggest the same bufotoxin resistance as their Australian congenerics (*Stegonotus cucullatus* and *Tropidonophis mairii*) which forage on invasive Cane Toads. Herein, we show the utility of short-read high coverage genomes, as well as improving the deficit of available squamate genomes with associated voucher specimens.

Keywords: Cane Toads, Squamates, Toxin resistance, Venom

Main Text

Introduction

Improvements of DNA sequencing and bioinformatics tools have increased scientists' ability to use molecular approaches to address a variety of evolutionary-related questions regarding species discovery, species limits, gene-flow analyses, gene expression, and selection (Lendemer *et al.*, 2020; Lum, Rheindt, & Chisholm, 2022; Nachman *et al.*, 2023). Squamates – amphisbaenians, lizards, and snakes – have become model systems for investigating such biological phenomena due to their high levels of intra- and intergroup variation (Gable *et al.*, 2023; Meiri, 2024; Title *et al.*, 2024). Investigations of evolutionary patterns and processes often implement a systematic approach using reduced representation datasets (*e.g.*, ultraconserved elements [UCEs], restriction site associated DNA sequencing [RADseq]) due to affordability and high success detecting phylogenetic signal between individuals and populations (Davey & Blaxter, 2010; Faircloth *et al.*, 2012; Palareti *et al.*, 2016; Blair *et al.*, 2019; Myers, McKelvy, & Burbrink, 2020; Bernstein *et al.*, 2023). The use of whole genomes in evolutionary biology has enabled a better understanding of underlying mechanisms that lead to extant diversity and factors that set lineages on different evolutionary trajectories (Martin *et al.*, 2018; Pasquesi *et al.*, 2018; Bravo, Schmitt, & Edwards, 2021; Del-Rio *et al.*, 2022; Ludington *et al.*, 2023). Despite their utility, there is currently a lack of high-quality genomes for squamates, and we are still very much in the infancy for widespread sequencing and application of squamate genomes. The increased sequencing of such genomes would provide valuable insight to comparative genomics, genome-phenotype relationships, and phylogenomics (Card, Jennings, & Edwards, 2023).

As whole genomes continue to become common practice in evolutionary biology, it is increasingly important to utilize datatypes that integrate with the associated molecular data (e.g., natural history observations; Title *et al.*, 2024). Museum voucher specimens that are used for whole genome sequencing also act as a valuable resource, linking the molecular data to the physical organism it came from and any natural history, environmental, morphological, or behavioral data associated with it. However, a large percentage of the currently available high coverage genomes across vertebrates lack corresponding voucher specimens. Recent examination of all available (~1,300) vertebrate genomes with >30X sequencing coverage found that only 11% of deposited genomes were accompanied by a voucher specimen (Buckner *et al.*,

2021), and with only 15% and 12% of available avian and reptilian genomes (>30X) having an associated voucher. This practice is problematic for many reasons: 1) genome sequencing data and genome assemblies are assumed to be correctly identified to species, leading to erroneous inferences in cases of taxonomic misidentification 2) some species with associated genome assemblies have undergone taxonomic revisions subsequent to sequencing, rendering repeatability impossible without a specimen to refer back to, 3) a lack of physical voucher removes traceable evidence linking the deposited genome to a legal collecting event, introducing possible legal ramifications or loss of data relevant to the specimen and genome. Additionally, GenBank entries rarely contain exhaustive sampling data such as local collaborators; such information is (or should always be) linked to deposited voucher specimens, and the loss of these data is a disservice to local collectors and collaborators who disproportionally are disconnected from research and resources derived from their contributions (Buckner *et al.*, 2021). Properly deposited genomes with associated museum vouchers improve the quality of research in any discipline that relies on open access genomic data, whether that is taxonomy, phylogenetics, or comparative genomics.

Linking genomes to voucher specimens increases the robustness of evolutionary and ecological inference by comparing newly collected/sequenced data with already-published molecular datasets. This has been successfully employed in many evolutionary scenarios, *i.e.*, investigating the genomic architecture for living at high altitudes (Lyu *et al.*, 2022), for adaptations against salinity (Rautsaw *et al.*, 2021), and resistance to tetrodotoxins (TTX; Montana, Ramírez-Castañeda, & Tarvin, 2023). An example, which we further elaborate on in this study, is analyzing open access genomes of understudied taxa and querying to see if species possess the genotype for immunity to the toxin of an introduced prey. One of the most studied species for observing toxin resistance are the cane toads of Australia (Phillips, Brown, & Shine, 2003). South American Cane Toads (*Rhinella marinus* [Linnaeus, 1758]) were introduced to Australia and Papua New Guinea during the early 1930s as an agricultural control measure for cane beetles, but instead caused an ecological disaster (Zug, 1975; Phillips *et al.*, 2006) when Cane Toads caused severe population declines by both consuming and poisoning native Australian fauna (Phillips *et al.*, 2003; Phillips, Brown, & Shine, 2004). They produce powerfully toxic cardiotonic steroids, known as bufotoxins (Akimova *et al.*, 2005; Keenan *et al.*, 2005; Bagrov,

Shapiro, & Fedorova, 2009) that kill non-resistant predators by blocking the sodium-potassium ATPase channels (NKAs hereafter) in cell membranes and causing cardiac-muscle immobilization (Soliev *et al.*, 2007). Cane Toads have been linked to severe declines in Australian snakes, with two exceptions being the Common Keelback, *Tropidonophis mairii* (Gray, 1841), and the Slatey-Grey Snake, *Stegonotus cucullatus*, which appear resistant to toad ingestion (Phillips *et al.*, 2003; Phillips & Shine, 2004). Cane Toad impacts on New Guinea fauna and bufotoxin resistance have never been investigated via genotyping. Sunda-Papuan Keelback snakes (Natricidae: *Tropidonophis*) comprise 20 species ranging in Australia, New Guinea, the Moluccas, and the Philippines – where native Asian toads of Bufonidae are found (*Ansonia* spp., *Ingerophrynus philippinicus*, *Pelophryne* spp.). The groundsnakes (Colubridae: *Stegonotus*) have a similar distribution, differing by a slightly further extension westward into Wallacea (Ruane *et al.*, 2018; Kaiser *et al.*, 2021). Genomic investigation of the *ATP1a3* paralog of the NKA α-subunit gene family has shown that toxin-resistant reptiles that consume bufotoxin-rich prey have glutamine-to-leucine and glycine-to-arginine substitutions at positions 111 and 120 (Ujvari *et al.*, 2012). These residues comprise the H1–H2 extracellular loop (amino acids 111-122 of *ATP1a3*), one of the primary bufotoxin binding sites for NKA inhibition. Sequences of the H1–H2 mRNA sequences for Australian *Tropidonophis mairii* and Australian *Stegonotus cucullatus* confirm the presence of the resistant H1–H2 phenotype (Ujvari *et al.*, 2015). These bufotoxin-resistant genotypes provide the genomic evidence for previous laboratory-based experiments proving that both *Tropidonophis mairii* and *Stegonotus cucullatus* in Australia are resistant to forced Cane Toad ingestion (Phillips *et al.*, 2003). Despite years of investigating bufotoxin resistance in many Australian snake lineages, *i.e.*, colubrids, elapids, natricids, and pythonids, bufotoxin resistance has never been investigated in New Guinean snakes and resistance is not known at this time.

Here, we present 18 advanced snake genome assemblies generated using recently collected high-quality tissue samples that have associated museum vouchers: *Acrochordus granulatus* (Schneider, 1799), *Aparallactus werneri* Boulenger, 1895, *Boaedon fuliginosus* (Boie, 1827), *Calamaria suluensis* Taylor, 1922, *Cerberus rynchops* (Schneider, 1799), *Grayia smithii* (Leach, 1818), *Imantodes cenchoa* (Linnaeus, 1758), *Mimophis mahfalensis* (Grandidier, 1867), *Oxyrhabdium leporinum* (Günther, 1858), *Pareas carinatus* Wager, 1830, *Psammodynastes*

pulverulentus (Boie, 1827), *Pseudoxenodon macrops* (Blyth, 1855), *Pseudoxyrhopus heterurus* (Jan, 1863), *Sibynophis collaris* (Gray, 1853), *Stegonotus admiraltiensis* Ruane, Richards, McVay, Tjaturadi, Krey,& Austin, 2017), *Toxicocalamus goodenoughensis* Roberts & Austin, 2020, *Trimeresurus albolabris* Gray, 1842, and *Tropidonophis doriae* (Boulenger, 1898). We use these genomes to show their utility in systematics and provide them as a genomic resource for the field of evolutionary biology. Additionally, we use select genomes of New Guinea snakes to provide evolutionary hypotheses on toxin resistance in New Guinea snakes for downstream investigations, highlighting the broader applicability of these resources outside systematics.

Methods

Biological Materials: All tissue samples were obtained from catalogued museum specimens from the Field Museum of Natural History (FMNH) or the Louisiana State University Museum of Natural Science (LSUMZ), and a single individual was used for each species.

Nucleic acid library preparation: DNA extraction was performed using the Qiagen DNAeasy genomic extraction kit using the standard process following manufacturer's protocol. Paired-end sequenced libraries were constructed using the Illumina TruSeq kit also according to the manufacturer's instructions.

DNA Sequencing, Genome Assembly, Completeness Assessment: The libraries were sequenced on an Illumina Hi-Seq platform in paired-end, 2 × 150bp format. The resulting fastq files were trimmed of adapter/primer sequence and low-quality regions with Trimmomatic v0.33 (Bolger, Lohse, & Usadel, 2014). The trimmed sequence was assembled by SPAdes v2.5 (Prjibelski *et al.*, 2020) followed by a finishing step using Zanfona (Kieras, O'Neill, & Pirro, 2021). Final genome statistics are presented in Table 1. In order to assess completeness of the genome assemblies, we conducted a Benchmarking Universal Single-Copy Orthologs (BUSCO) assessment within the program *compleasm* (Huang & Li, 2023). *compleasm* uses a given BUSCO database and employs *miniprot* (Li, 2023) as the default protein-to-genome aligner. For a BUSCO reference, *compleasm* benchmarked the 18 snake genomes herein against the available Vertebrata Ortholog Database v10.

Reduced Representation Mining for Phylogenetics: For *in silica* sequence capture of ultraconserved elements (UCEs; Faircloth *et al.*, 2012) and Squamate Conserved Loci version 2 probeset (SqCL; Singhal *et al.*, 2017), we used *phyluce* v1.6 (Faircloth, 2015). The SqCL probeset comprises a combination of anchored hybrid enriched loci (AHEs; Lemmon, Emme, & Lemmon, 2012), UCEs, and traditional phylogenetic gene loci. For simplicity, the UCE-only dataset is referred herein as simply UCEs, and the SqCL probeset as SqCL instead of its primary components: UCEs, AHEs and traditional Sanger loci. For UCE calling, we followed the UCE mining tutorial III that instructs proper UCE mining for previously published or assembled genomes. We first converted all final Zanfona genome assemblies to 2bit format, and then searched the 2bit assemblies for UCEs within the Tetrapods 5K UCE probeset. For SqCL marker mining, the same approach was taken, but the headers for each SqCL bait were modified to allow *phyluce* to parse and select out the SqCL loci. We then aligned all recovered UCE and SqCL loci with MAFFT (Katoh & Standley, 2013). For phylogenetic analyses, we filtered our UCEs and SqCL with *phyluce* and created 75% completeness concatenated alignments, one per probeset, selecting only loci that include 75% or more of represented taxa in our dataset. Concatenated alignments were input into IQ-TREE 2.0 (Minh *et al.*, 2020) for maximum likelihood tree inference to compare to previous studies directly investigating snake familial phylogenetic relationships (Zaher *et al.*, 2019; Burbrink *et al.*, 2020). We ran IQ-TREE with both alignments using the MFP (ModelFinder Plus) option that performs an exhaustive ModelFinder (Lanfear *et al.*, 2012) search for the best fit substitution model then automatically begins inference with the best fit model . We used the default option of n=1000 ultrafast bootstrap replications to reconcile with the best tree found during maximum likelihood tree search (Hoang *et al.*, 2018).

Toxin Resistance Gene Mining: To show the utility of *de novo* short read genomes from non-model and rare taxa, we mined the genomes of two species, *Stegonotus admiraltiensis* and *Tropidonophis doriae*, for the genotype responsible for either bufotoxin sensitivity or resistance. To date, the Burmese Python genome (*Python bivittatus* – Accession No. GCF_000186305.1) is one of the highest quality annotated genomes for any snake (Castoe *et al.*, 2011). For mining our two New Guinea snake genomes, we used the annotated *ATP1a3* protein sequence from *Python*

bivittatus, a species that is susceptible to bufotoxin poisoning (Mohammadi *et al.*, 2016). We used the *tblastn* function within NCBI's BLAST. We set the *Python bivittatus ATP1a3* gene as the query and *tblastn* against the deposited *Stegonotus admiraltiensis* and *Tropdinophis doriae* genomes. We then compared the *Python bivittatus* query results and found the highest coverage result overlapping with the H1–H2 region. We then aligned this best-fit sequence from the query with the GenBank *ATP1a3* H1–H2 sequences for *Stegonotus cucullatus* (KP238138.1) and *Tropidonophis mairii* (KP238142.1) from Australia.

Results

Genome sequencing: Raw sequence data and genome assemblies were deposited into GenBank for public access. See Tables 1 and 2 for accession information and genome assembly statistics for the dataset. BUSCO completeness via *compleasm* are available in Table 2. The mean and standard deviation of single copy complete genes (S in *compleasm* output) recovered in the assemblies was 2,468±229 loci. Out of the total 3,354 loci available in the Vertebrata Ortholog Database v10 used as a reference, this represents an average BUSCO score of 73.6%.

Phylogenetics: We successfully mined UCEs, AHEs, and traditional Sanger loci from the new genome assemblies. We recovered a mean of 3,326 UCEs and 4,743 SqCLs per assembly (Supplement Table 1). IQ-TREE inferred 100% congruent phylogenies for the UCE and SqCL alignments (Fig. 1). Compared to recent family-level snake phylogenies (Zaher *et al.*, 2019; Burbrink *et al.*, 2020), both phylogenies for Caenophidia inferred from our genome assemblies are similar. Differences between our phylogeny and those that were previously published differ by missing taxa, so an exhaustive comparison between our phylogenies and others is difficult. Despite this, we have successfully shown the utility of short read genomes for phylogenomics using multiple probesets commonly used for squamate systematics.

Toxin resistance: *tblastn* using the bufotoxin-susceptible *Python bivittatus* genotype against the two Papua New Guinea snake genomes recovered the targeted locus for both genomes. For *Stegonotus admiraltiensis*, the exon coding the H1–H2 extracellular loop was recovered on scaffold number 4,766, spanning bases 54,077 – 53,988 (3'–5' direction). For *T. doriae*, the exon

was found on scaffold 4,558 spanning bases 9,790 – 9,701 (3'–5' direction). When translated and aligned with *P. bivittatus* and the two bufotoxin-resistant sequences for *S. cucullatus* and *T. mairii* from Australia, the retrieved exons from both these previously uninvestigated New Guinea taxa showed that they both possess the genotype for bufotoxin resistance, specifically a leucine (L) at position 111 versus a glutamine (Q), and an arginine (R) at position 120 versus a glycine (G) (Fig. 2; see also (Ujvari *et al.*, 2015). This comprises the first evidence of bufotoxin resistance in New Guinea snakes, despite evolving allopatrically from any toad species until human-mediated introduction in the early 20th century.

Discussion

Evolutionary biology research using non-model vertebrate systems is becoming more and more common, and in parallel, genomic resources are increasing at rapid rates with a decrease in sequencing costs, paving the way to test new hypotheses and investigate novel systems (Haussler *et al.*, 2009; Meadows & Lindblad-Toh, 2017; Rhie *et al.*, 2021). Here, we provide 18 new genomes which represent ~50% of all snake families, and nearly 100% of caenophidian snake families (Zaher *et al.*, 2019; Burbrink *et al.*, 2020). These genomes can be used as resources for a variety of disciplines in evolutionary biology, such as broad-scale systematics, phylogenomics, biogeography, and, as shown here, phenotype patterns and evolution.

The average BUSCO scores of these genomes is lower than a Reference Sequence genome (RefSeq) assemblied using a three-prong and expensive sequencing approach: long-reads (PacBio or Oxford Nanopore), short-reads for genome "polishing" (Illumina-based genome sequencing), followed by transcriptome-based annotation. The lower completion scores (Table 2) are primarily because these 18 assemblies are all solely Illumina short-read based. In addition, these genomes are also currently published on Genbank as Draft 1 assemblies. Despite the absence of long-read sequencing such as Oxford Nanopore or PacBio, these genomes will continue to improve in BUSCO completeness scores as sequential drafts are updated to these accessions due to additional *in silica* curation and read-merging by the genome depositors (Stacy Pirro – Iridian Genomes). Despite their current BUSCO score, we have shown herein the wide application that these genomes already serve even in their current first-draft state.

Reduced representation datasets using probe sets have become widely used in systematics, allowing for denser taxonomic sampling, higher throughput, and lower sequencing costs compared to long read whole genomes. The use of probe sets in systematics has become useful for balancing costs of sequencing with project sample number and the amount of informative data received (Faircloth *et al.*, 2012; Lemmon *et al.*, 2012; Singhal *et al.*, 2017; Karin *et al.*, 2020). While such datasets are extremely useful for testing hypotheses in evolutionary biology, such as species boundaries and diversification scenarios (Skipwith & Oliver, 2023), these targeted loci are spread throughout the genome, often without reference genomes to understand the physical location and respective patterns of each locus in the genome. Sequencing more continuous sections of the genome, or the entire genome itself, can provide a better understanding of genome architecture and the mechanisms that underpin genomic patterns and evolution, while still enabling researchers to target specific research aims for systematic and population genomic studies in which reduced representation data were used (Lou *et al.*, 2021).

Systematic studies aim to identify the evolutionary relationships and draw inference on biogeography, species diversity, conservation efforts, and, sometimes, identify regions of the genome relevant for more in-depth evolutionary studies (Singhal *et al.*, 2021; Pavón-Vázquez *et al.*, 2022; Shaffer *et al.*, 2022; Mochales-Riaño *et al.*, 2024). We emphasize the use of whole-genomes to broaden systematic studies towards targeting finer-scaled biological aims of the study system, such as what we show here with *Stegonotus admiraltiensis* and *Tropidonophis doriae*. A particular genotype of the *ATP1a3* gene is needed for snakes (and other squamates) to safely ingest toxic toads (Anura: Bufonidae) due to endogenous bufotoxins produced in toads. This has been observed in thamnophiines (*Thamnophis*; Mohammadi *et al.*, 2016, 2017), and we now confirm this for two other colubrids, *Stegonotus admiraltiensis* and *Tropidonophis doriae*. Interestingly, while evolutionarily naïve to toads and their toxins, *Stegonotus admiraltiensis* within the last 100 years has been faced with the highly toxic introduced Cane Toads across Manus Island (Fig. 2). The *Tropidonophis doriae* specimen collected herein (LSUMZ 129280) was collected from a mid-elevation (800 meters asl) field site. This population currently exists in complete allopatry with introduced Cane Toads due to the elevational barrier for these invaders (~300 meters asl; Zug, 1975). Despite differences in current sympatry-or-allopatry with Cane Toads between these two New Guinea snake endemics, both *Stegonotus admiraltiensis* and

Tropidonophis doriae possess the bufotoxin-resistant genotype (Fig. 2). Our example here can be compared with other systems that contain snake lineages that overlap with toxic toads but lack genotypes that likely lead to toxin-resistance (e.g., boids, lamprophiids; Marshall, 2017). Many other snake taxa act as opportunistic models to investigate the evolution of toxin-resistance (or susceptibility to bufotoxins), and morphological, behavioral, and physiological data exists (Phillips *et al.*, 2003; Phillips, 2004; Pearson *et al.*, 2014; Llewelyn *et al.*, 2018), along with evidence of non-genotypic mechanisms related to toxin-resistance (Mohammadi *et al.*, 2017a) that can be supplemented by whole-genome datasets.

Two of the species included in our dataset were described within the last 10 years and their assemblies are sequenced from contemporary tissues cryogenically stored in ethanol, being removed from the holotype specimens at time of preparation (*Stegonotus admiraltiensis* and *Toxicocalamus goodenoughensis*; Ruane *et al.*, 2018; Roberts & Austin, 2020). For museum scientists focusing their collecting efforts in poorly explored areas with high potential for new species discovery, we strongly recommend the incorporation of a whole-genome assembly voucher. Similar to how the optimal whole specimen is chosen to represent the holotype for a new species, museum scientists should consider submitting a sample from the best representative for whole-genome sequencing to further extend the utility of the specimen. This recommendation would previously qualify as exclusive to only large institutions due to whole-genome sequencing cost, but this is no longer the case. The average cost for the sequencing of these Illumina short-read high coverage genomes was ~$300/sample (Stacy Pirro, Iridian Genomes). Even if whole-genome sequencing may be outside the research questions of the specimen, deposition of whole-genomes from type material, or even topotypic voucher material, improves taxonomy and saves both money and resources for future field collectors and researchers. Tissue collections of museums are invaluable, but also nonrenewable, resources (Sheldon & Dittmann, 1997). As of 2024, once a freshly preserved tissue (ethanol, liquid nitrogen, *etc*.) is exhausted from a specimen, whole-genome quality tissue samples cannot be retrieved from the specimen. Our techniques and applications for targeted sequence capture of formalin-fixed tissues are improving and broadening (Bernstein & Ruane, 2022; Bernstein *et al.*, 2023), but the preferred sample is still freshly preserved tissue. Incorporating whole-genome sequencing as a part of the cataloging and processing pipeline of new species and rare collections will expand our

knowledge and collaboration within this field, protect and extend the longevity of current tissue stocks in collections, and save collecting resources. For example, during manuscript preparation, the above data contributed to researchers studying genome evolution in Asian snakes which led to the recent description of a new family, Psammodynastidae, based largely on *in silica* loci mining of the *Psammodynastes pulverulentus* genome assembly presented above (Das *et al.*, 2024).

We understand that whole genome sequencing is not always financially feasible and is not always necessary for fine-scaled evolutionary questions such as determining taxonomic placement or reconstructing a well-resolved phylogeny. Indeed, it may be more cost-effective to sequence from targeted probe sets for such projects. However, we provide these genomes as resources for researchers aiming to study related taxa in a systematic context or for comparative purposes in broader investigations of snake evolution. A wealth of evolutionary information is lost when using target capture approaches or select loci, leading to gaps in our knowledge of what has led to extant diversity. The genomes we provide will contain greater degrees of evolutionary history, which can still be used for finer-scaled questions, and we hope researchers use the resources provided here for both fine- and broad-scale squamate and evolutionary research. In addition, we hope these new assemblies can persuade other laboratories and research institutes who are field collecting to consider selecting best-samples with whole-specimen vouchers as potential genome vouchers for all researchers to use.

Inference of congruent phylogenies with robust support and coupled with fine-scale application towards toxin resistance prove the utility towards broad applications of these 18 newly deposited genome assemblies. These 18 assemblies have been sequenced from under-represented snakes in distinct families that vary in their life history traits. These assemblies increase the growing genomic resources available for snakes and improve upon the dearth of available snake genomes with associated museum voucher material (Table 1). Buckner *et al*., 2021 presented many reasons why genomes *sans* vouchers introduce more problems than benefits to genomics and evolutionary science. When depositing whole-genome assemblies (or even single locus datasets on GenBank), the linking of vouchered material to the sequence data broadens the application potential, increasing the value of both assembly and vouchered specimen.

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Table 1. Genome assembly statistics for the new 18 snake genomes presented herein.

Family	Species	Catalog number	Raw reads	Genome assembly	Genome size	Total ungapped length	No. scaffolds	Scaffold N50	Scaffold L50	No. contigs	Contig N50	Contig L50	GC%	Genome coverage
Acrochordidae	*Acrochordus granulatus*	FMNH 216296	SRR18186315	JANHZU000000000	1.3 Gb	1.3 Gb	184,515	156.8 kb	2,274	356,517	7 kb	56,464	38.0%	120.0X
Atractaspididae	*Aparallactus werneri*	FMNH 251842	SRR18186078	JANHFQ000000000	1.3 Gb	1.3 Gb	577,532	40.5 kb	4,756	792,783	2.7 kb	134,499	40.0%	90.0X
Lamprophiidae	*Boaedon fuliginosus*	FMNH 251328	SRR18185936	JANHGC000000000	1.4 Gb	1.4 Gb	583,668	62.6 kb	4,025	753,228	4 kb	93,940	40.5%	105.0X
Calamariidae	*Calamaria suluensis*	FMNH 273639	SRR18186341	JANHFP000000000	1.3 Gb	1.2 Gb	431,810	37 kb	4,852	595,453	3.5 kb	100,656	40.0%	105.0X
Homalopsidae	*Cerberus rynchops*	FMNH 250126	SRR19075914	JANHFJ000000000	1.3 Gb	1.2 Gb	415,657	72.1 kb	3,580	627,335	3.4 kb	107,820	39.0%	130.0X
Grayiidae	*Grayia smithii*	LSUMZ 44406	SRR18191648	JANHFM000000000	1.4 Gb	1.4 Gb	424,630	93.2 kb	3,291	613,975	4.4 kb	88,764	40.0%	100.0X
Dipsadidae	*Imantodes cenchoa*	FMNH 282648	SRR18185477	JANHGD000000000	1.4 Gb	1.3 Gb	605,251	52.4 kb	4,488	830,518	2.9 kb	132,253	40.5%	95.0X
Psammophiidae	*Mimophis mahfalensis*	FMNH 259984	SRR18183298	JANHFR000000000	1.5 Gb	1.4 Gb	710,676	46.8 kb	4,947	905,380	3.4 kb	120,140	41.0%	85.0X
Cyclocoridae	*Oxyrhabdium leporinum*	FMNH 278897	SRR18183299	JANHFL000000000	1.3 Gb	1.2 Gb	245,550	137.7 kb	2,380	369,505	8.4 kb	41,316	39.5%	85.0X
Pareidae	*Pareas carinatus*	FMNH 255233	SRR18183318	JANHFO000000000	1.4 Gb	1.4 Gb	421,728	92.3 kb	3,351	584,266	5.2 kb	79,601	40.0%	90.0X
Pseudaspidae	*Psammodynastes pulverulentus*	FMNH 273629	SRR19070322	JAOYMU000000000	1.5 Gb	1.2 Gb	103,289	69.3 Mb	7	378,801	4.6 kb	76.661	39.5%	105.0X
Pseudoxenodontidae	*Pseudoxenodon macrops*	FMNH 255568	SRR18184335	JANHFT000000000	1.4 Gb	1.3 Gb	338,712	114 kb	2,813	491,605	6 kb	61,062	40.0%	105.0X
Pseudoxyrhophidae	*Pseudoxyrhopus heterurus*	FMNH 259987	SRR19067745	JANHJO000000000	1.3 Gb	1.2 Gb	370,324	103.6 kb	2,867	577,929	4.1 kb	83,963	39.5%	115.0X
Sibynophiidae	*Sibynophis collaris*	FMNH 255570	SRR18355959	JANHFK000000000	1.3 Gb	1.3 Gb	459,228	84.2 Kb	3314	669,060	3.5 kb	100,747	39.5%	800.0X
Colubridae	*Stegonotus admiraltiensis*	LSUMZ 93597	SRR18191894	JANHZT000000000	1.5 Gb	1.5 Gb	642,501	80 kb	3,857	837,256	4.1 kb	101,236	40.5%	95.0X
Elapidae	*Toxicocalamus goodenoughensis*	LSUMZ 98043	SRR18191708	JANHFN000000000	1.3 Gb	1.3 Gb	313,041	110.8 kb	2,809	457,766	6.1 kb	59,501	39.0%	110.0X
Viperidae	*Trimeresurus albolabris*	FMNH 255254	SRR18183273	JANHFS000000000	1.2 Gb	1.2 Gb	317,626	113.2 kb	2,638	497,879	4.7 kb	70,780	39.0%	90.0X
Natricidae	*Tropidonophis doriae*	LSUMZ 129280	SRR18210580	JAPKID000000000	1.3 Gb	1.3 Gb	693,335	3.5 kb	111,586	703,744	3.4 kb	114,448	40.0%	100.0X

Table 2. Genome assembly completeness predicted from *compleasm* for the new 18 snake genomes. The output of *compleasm* is summarized as five values: S (single copy complete genes), D (duplicated complete genes), F (fragmented genes which only a portion is present and the rest cannot be aligned, subclass 1), I (fragmented genes in which a section of the gene aligns to one position in the assembly, while the remaining part aligns to another position, subclass 2), and M (missing genes). Values are reported as the gene number recovered followed by the percentage of recovered genes within total Vertebrata BUSCO genes database (n = 3,354).

Family	Species	Catalog number	Raw reads	Genome assembly	S	D	F	I	M
Acrochordidae	*Acrochordus granulatus*	FMNH 216296	SRR18186315	JANHZU000000000	2,935 (87.5%)	4 (0.1%)	305 (9.1%)	2 (0.1%)	108 (3.2%)
Atractaspididae	*Aparallactus werneri*	FMNH 251842	SRR18186078	JANHFQ000000000	2,167 (64.6%)	8 (0.2%)	667 (19.9%)	8 (0.2%)	504 (15.0%)
Lamprophiidae	*Boaedon fuliginosus*	FMNH 251328	SRR18185936	JANHGC000000000	2,275 (67.8%)	10 (0.3%)	663 (19.8%)	6 (0.2%)	400 (11.9%)
Calamariidae	*Calamaria suluensis*	FMNH 273639	SRR18186341	JANHFP000000000	2,137 (63.7%)	7 (0.2%)	683 (20.4%)	10 (0.3%)	517 (15.4%)
Homalopsidae	*Cerberus rynchops*	FMNH 250126	SRR19075914	JANHFJ000000000	2,274 (67.8%)	8 (0.2%)	650 (19.4%)	9 (0.3%)	413 (12.3%)
Grayiidae	*Grayia smithii*	LSUMZ 44406	SRR18191648	JANHFM000000000	2,384 (71.1%)	10 (0.3%)	581 (17.3%)	6 (0.2%)	373 (11.12%)
Dipsadidae	*Imantodes cenchoa*	FMNH 282648	SRR18185477	JANHGD000000000	2,281 (68.0%)	9 (0.3%)	637 (19.0%)	8 (0.2%)	419 (12.5%)
Psammophiidae	*Mimophis mahfalensis*	FMNH 259984	SRR18183298	JANHFR000000000	2,411 (71.9%)	6 (0.2%)	626 (18.7%)	8 (0.2%)	303 (9.0%)
Cyclocoridae	*Oxyrhabdium leporinum*	FMNH 278897	SRR18183299	JANHFL000000000	2,663 (79.4%)	4 (0.1%)	483 (14.4%)	10 (0.3%)	194 (5.8%)
Pareidae	*Pareas carinatus*	FMNH 255233	SRR18183318	JANHFO000000000	2,449 (73.0%)	11 (0.3%)	608 (18.1%)	8 (0.2%)	278 (8.3%)
Pseudaspidae	*Psammodynastes pulverulentus*	FMNH 273629	SRR19070322	JAOYMU000000000	2,802 (83.5%)	14 (0.4%)	329 (9.8%)	5 (0.2%)	204 (6.1%)
Pseudoxenodontidae	*Pseudoxenodon macrops*	FMNH 255568	SRR18184335	JANHFT000000000	2,413 (71.9%)	11 (0.3%)	622 (18.9%)	8 (0.2%)	300 (8.9%)
Pseudoxyrhophidae	*Pseudoxyrhopus heterurus*	FMNH 259987	SRR19067745	JANHJO000000000	2,415 (72.0%)	4 (0.1%)	591 (17.6%)	6 (0.2%)	338 (10.1%)
Sibynophiidae	*Sibynophis collaris*	FMNH 255570	SRR18355959	JANHFK000000000	2,361 (70.4%)	6 (0.2%)	608 (18.1%)	6 (0.2%)	373 (11.12%)
Colubridae	*Stegonotus admiraltiensis*	LSUMZ 93597	SRR18191894	JANHZT000000000	2,566 (76.5%)	6 (0.2%)	529 (15.8%)	6 (0.2%)	247 (7.36%)
Elapidae	*Toxicocalamus goodenoughensis*	LSUMZ 98043	SRR18191708	JANHFN000000000	2,539 (75.7%)	4 (0.1%)	554 (16.52%	7 (0.2%)	250 (7.5%)
Viperidae	*Trimeresurus albolabris*	FMNH 255254	SRR18183273	JANHFS000000000	2,461 (73.4%)	6 (0.2%)	586 (17.5%)	8 (0.2%)	293 (8.7%)
Natricidae	*Tropidonophis doriae*	LSUMZ 129280	SRR18210580	JAPKID000000000	2,884 (86.0%)	7 (0.2%)	274 (8.2%)	1 (0.03%)	188 (5.6%)

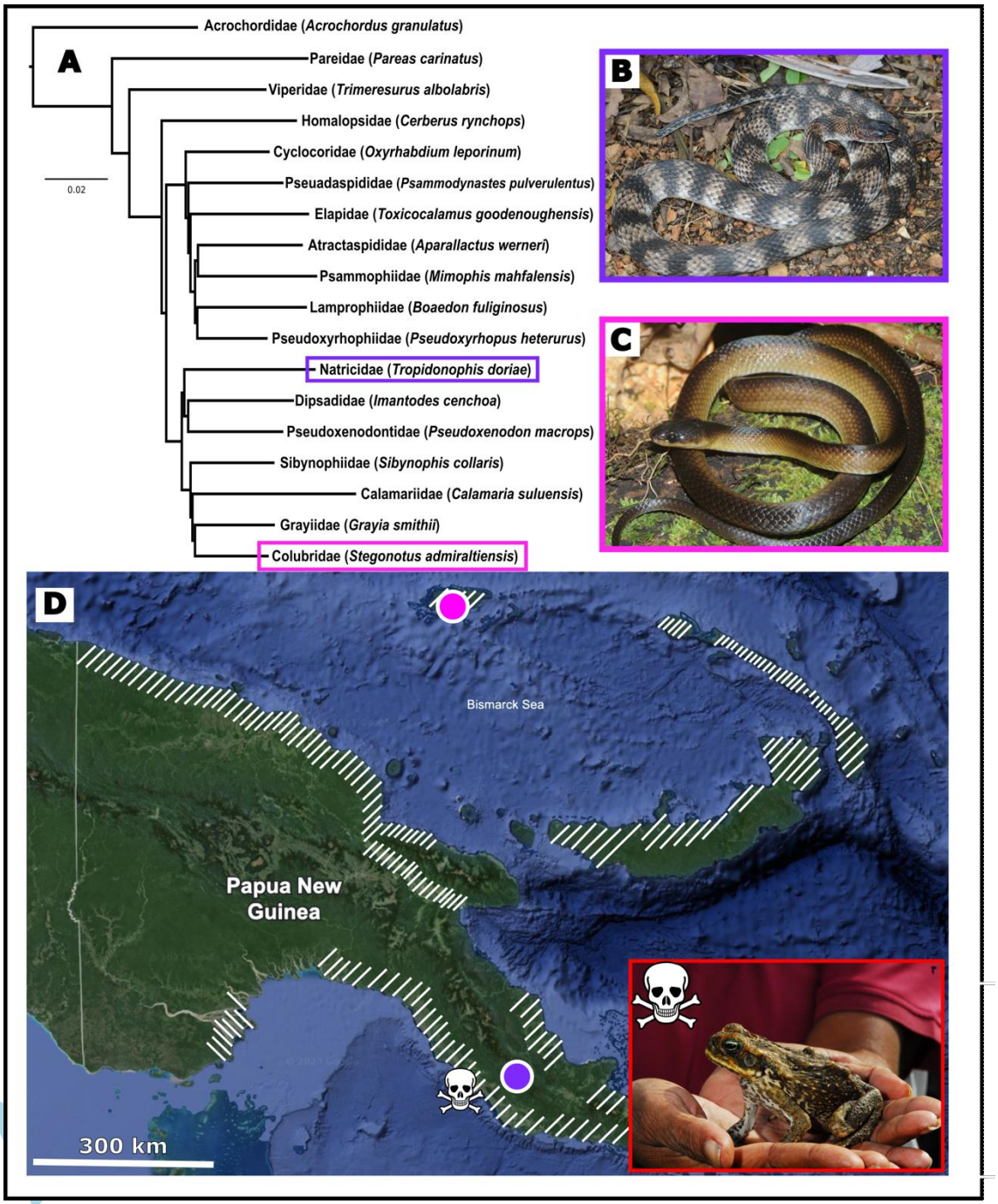

Figure 1. A) Inferred phylogeny in IQ-TREE from UCE and SqCL mining of the new 18 snake genomes (Serpentes: Caenophidia). The topology above represents the UCE phylogeny. All inferred nodes were strongly supported with ultrafast bootstrap support of 100. B) A photo in life of *Tropidonophis doriae* (LSUMZ 129280 – purple outline), a topotypic voucher collected near the type locality. C) A photo in life of *Stegonotus admiraltiensis* (LSUMZ 93597 – pink outline), a species endemic to Papua New Guinea and represented in our dataset by the whole-genome assembly from the holotype of this species. D) Map of Papua New Guinea, the largest tropical island in the world. White hatching represents current Cane Toad, *Rhinella marinus*, distribution according to Zug (1975) and VertNet query. E) Cane Toad, *Rhinella marinus*, collected from the country capitol, Port Moresby. The purple, pink, and skull-and-crossbones mark the localities of *Tropidonophis doriae* (B - purple), *Stegonotus admitaltiensis* (C - pink) and the Cane Toad, respectively.

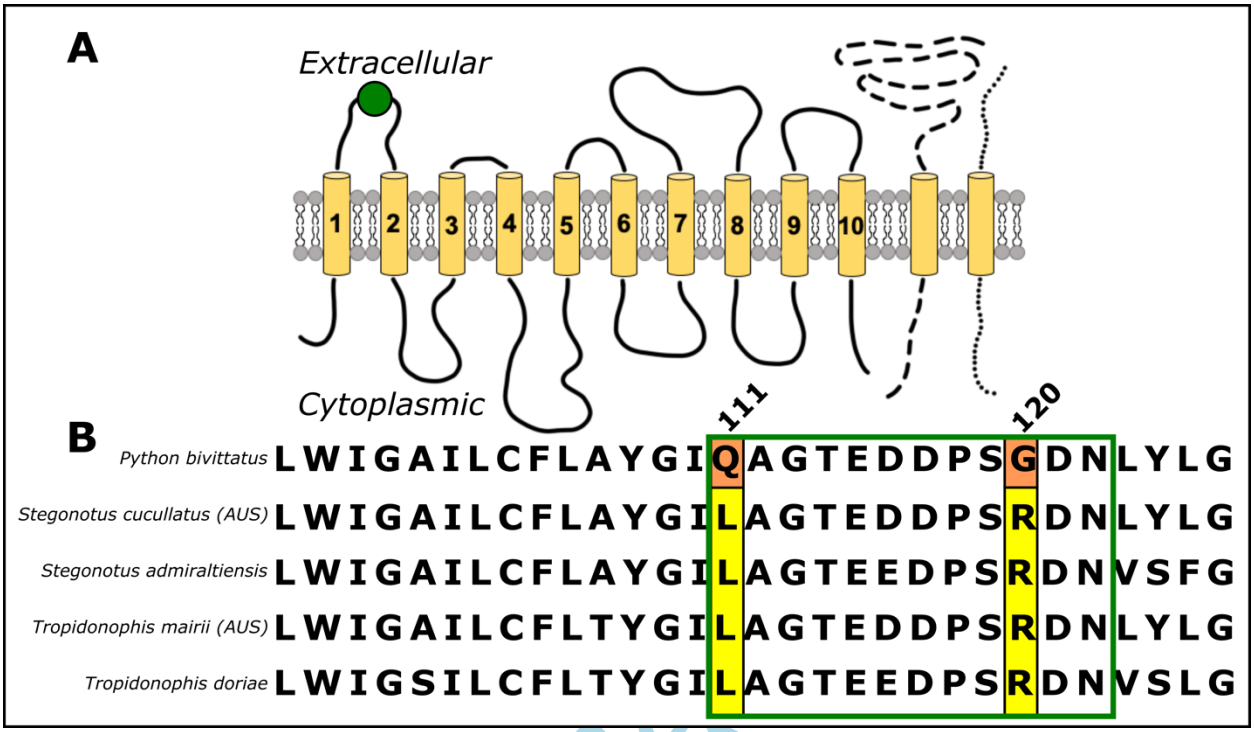

Figure 2. A) Structure of the eukaryotic NKA channel (modified after Bagrov *et al*., 2009) showing the three subunits: the α subunit (solid line) with 10 transmembrane proteins, the β subunit (dashed line) with one transmembrane protein, and the γ subunit (dotted line) with one transmembrane protein. Three extracellular bufotoxin-binding sites are known, but only the H1–H2 extracellular loop (green circle) has been investigated in reptiles. B) The protein alignment of the H1–H2 extracellular loop for *Python bivittatus*, *Stegonotus cucullatus* from Australia, *Stegonotus admiraltiensis*, *Tropidonophis mairii* from Australia, and *Tropidonophis doriae* showing the presence of either the bufotoxin-susceptible genotypes at amino acid 111 and 120 (orange – *Python bivittatus* only) or the resistant phenotype (yellow – *Stegonotus* spp. and *Tropidonophis* spp.).