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Genome Resources

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Whole snake genomes from eighteen families of snakes (Serpentes: Caenophidia) and their applications to systematics

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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 ultraconserved elements (UCEs) and anchored hybrid enriched loci (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 the 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 (*S. admiraltiensis* and *T. 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 australis* 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.

Key words: Cane Toads, squamates, toxin resistance, venom

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 et al. 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 and Blaxter 2010; Faircloth et al. 2012; Palareti et al. 2016; Blair et al. 2019; Myers, et al. 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

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(Martin et al. 2018; Pasquesi et al. 2018; Bravo et al. 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 of 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 et al. 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 >30× 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 (>30×) 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, and 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, that is, 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 et al. 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 et al. 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 et al. 2004). They produce powerfully toxic cardiotonic steroids, known as bufotoxins (Akimova et al. 2005; Keenan et al. 2005; Bagrov et al. 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 Australian Groundsnake, *Stegonotus australis* (Günther, 1872), which appear resistant to toad ingestion (Phillips et al. 2003; Phillips and 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 to 122 of *ATP1a3*), one of the primary bufotoxin-binding sites for NKA inhibition. Sequences of the H1–H2 mRNA sequences for Australian *T. mairii* and Australian *S. australis* 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 *T. mairii* and *S. australis* in Australia are resistant to forced Cane Toad ingestion (Phillips et al. 2003). Despite years of investigating bufotoxin resistance in many Australian snake lineages, that is, 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 and 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 cataloged 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 the 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 × 150 bp format. The resulting fastq files were trimmed of adapter/primer sequence and low-quality regions with Trimmomatic v0.33 (Bolger et al. 2014). The trimmed sequence was assembled by SPAdes v2.5 (Prjibelski et al. 2020) followed by a finishing step using Zanfona (Kieras et al. 2021). Final genome statistics are presented in Table 1. In order to assess the completeness of the genome assemblies, we conducted a Benchmarking Universal Single-Copy Orthologs (BUSCO) assessment within the program *compleasm* (Huang and 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 et al. 2012), UCEs, and traditional phylogenetic gene loci. For simplicity, the UCE-only dataset is referred to 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 and 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 and 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 the 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, *S. admiraltiensis* and *T. 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 *P. 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 *P. bivittatus ATP1a3* gene as the query and *tblastn* against the deposited *S. admiraltiensis* and *Tropidonophis doriae* genomes. We then compared the *P. 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 *S. australis* (labeled in Genbank as *S. cucullatus* - KP238138.1) and *T. 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 (Supplementary 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 *P. bivittatus* genotype against the two Papua New Guinea snake genomes recovered the targeted locus for both genomes. For *S. admiraltiensis*, the exon coding the H1–H2 extracellular loop was recovered on scaffold number 4,766, spanning bases 54,077 to 53,988 (3′–5′ direction). For *T. doriae*, the exon was

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

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

* formerly Pseudaspididae

Table 2. Genome assembly completeness predicted from *compleasm* for the new 18 snake genomes.

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%)
Psammodynastidae*	*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%)
Pseudoxyrhophiidae	*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%)

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).
* formerly Pseudaspididae

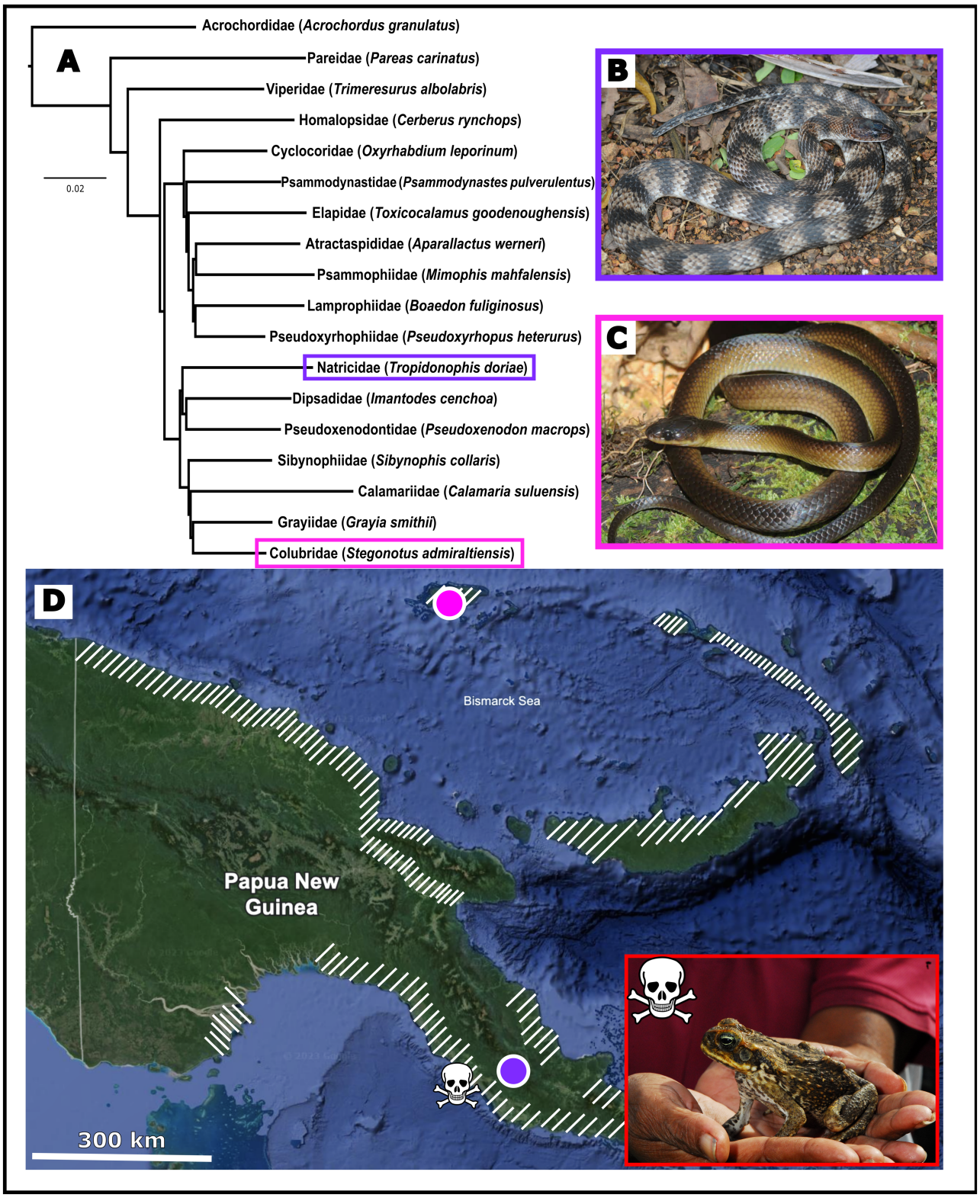

Fig. 1. A) Inferred phylogeny in IQ-TREE from UCE and SqCL mining of the new 18 snake genomes (Serpentes: Caenophidia). The topology above represents the UCE phylogeny. All inferred nodes were strongly supported with ultrafast bootstrap support of 100. B) A photo in the life of *Tropidonophis doriae* (LSUMZ 129280—Natricidae), a topotypic voucher collected near the type locality. C) A photo in the life of *Stegonotus admiraltiensis* (LSUMZ 93597—Colubridae), 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. The inset within the map shows a Cane Toad, *R. marinus*, collected from the country capital, Port Moresby. The purple, pink, and skull-and-crossbones mark the localities of *T. doriae* (B—purple circle on Papua New Guinea mainland), *S. admitaltiensis* (C—pink circle in Bismarck Sea), and the Cane Toad, respectively.

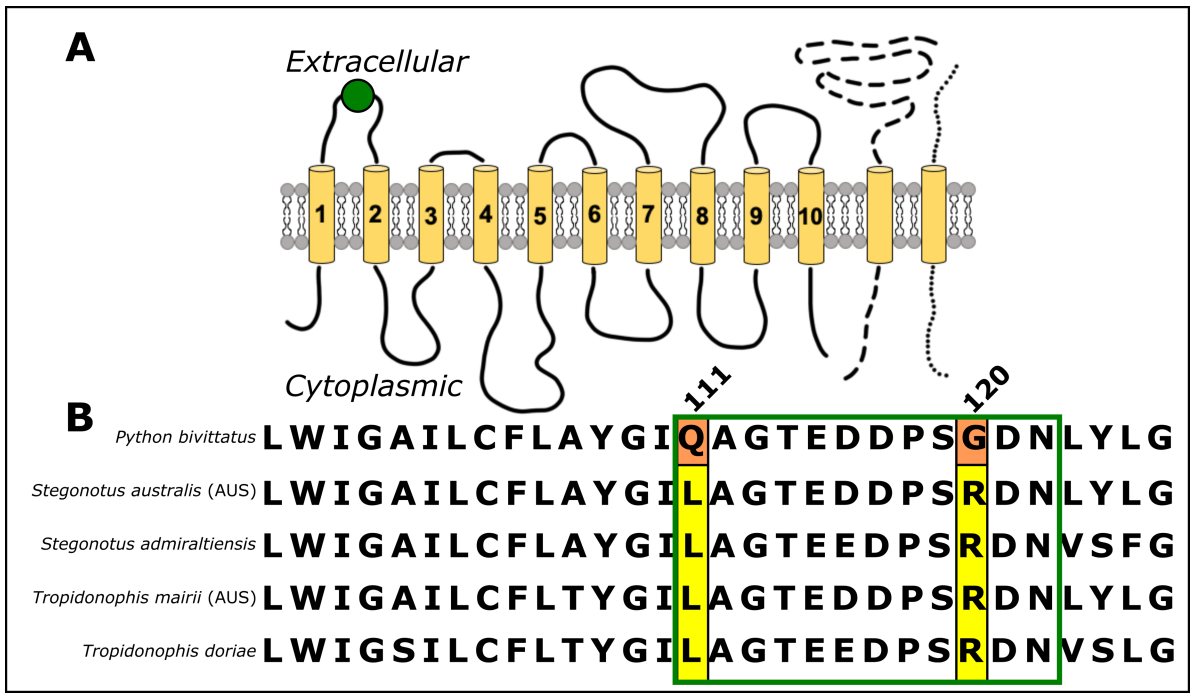

Fig. 2. A) Structure of the eukaryotic NKA channel (modified after Bagrov et al. 2009) showing the three subunits: the α subunit (solid line) with 10 transmembrane proteins, the β subunit (dashed line) with one transmembrane protein, and the γ subunit (dotted line) with one transmembrane protein. Three extracellular bufotoxin-binding sites are known, but only the H1–H2 extracellular loop (denoted by circle) has been investigated in reptiles. B) The protein alignment of the H1–H2 extracellular loop for *Python bivittatus*, *Stegonotus australis* 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—*P. bivittatus* only) or the resistant phenotype (yellow—*Stegonotus* spp. and *Tropidonophis* spp.).

found on scaffold 4,558 spanning bases 9,790 to 9,701 (3′–5′ direction). When translated and aligned with *P. bivittatus* and the two bufotoxin-resistant sequences for *S. australis* 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 glutamine (Q), and 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 and 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 a 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 the 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 and 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 inferences on biogeography, species diversity, and 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 *S. admiraltiensis* and *T. 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, 2017a, 2017b), and we now confirm this for two other colubrids, *S. admiraltiensis* and *T. doriae*. Interestingly, while evolutionarily naïve to toads and their toxins, *S. admiraltiensis* within the last 100 years has been faced with the highly toxic introduced Cane Toads across Manus Island (Fig. 1). The *T. doriae* specimen collected herein (LSUMZ 129280) was collected from a mid-elevation (800 m asl) field site. This population currently exists in complete allopatry with introduced Cane Toads due to the elevational barrier for these invaders (~300 m asl; Zug 1975). Despite differences in current sympatry-or-allopatry with Cane Toads between these two New Guinea snake endemics, both *S. admiraltiensis* and *T. 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 exist (Phillips et al. 2003, 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 the time of preparation (*S. admiraltiensis* and *T. goodenoughensis*; Ruane et al. 2018; Roberts and 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 and Dittmann 1997). As of 2024, once 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 and 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 *P. 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 the 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 specimens.

Supplementary material

Supplementary material is available at *Journal of Heredity* Journal online.

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Data availability statement

The data underlying this article are available in the Genbank Nucleotide Database at https://www.ncbi.nlm.nih.gov/bioproject/?term=Sara+Ruane, and can also be accessed individually using either the Sequence Read Archive or genome assembly accession numbers in Table 1.

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