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## **Abstract**

Colubridae represents the most phenotypically diverse and speciose family of snakes, yet no well-assembled and annotated genome

compounds of their prey, specifically in toxic newts of the genus *Pleurodeles*. Given this unique adaptation, garter snakes have become an important model for studying the evolution of toxin resistance and coevolutionary dynamics of predator–prey arms races (Brodie and Brodie 1999; Brodie et al. 2002; Feldman et al. 2009; McGlothlin et al. 2016).

Here, we sequenced and annotated the genome of the garter snake (*Thamnophis sirtalis*) and used this genome and related resources to address broad questions related to vertebrate genome evolution and garter snake biology. We examined molecular evolution across gene families that underlie unique and interesting biological features of garter snakes related to toxin resistance and venom, as well as olfactory and visual systems. We also compared evolutionary patterns of genome composition and structure (repeat content, microRNAs, and sex chromosomes) in garter snakes in the context of other amniote genomes, identified genes that have likely been under positive selection in the garter snake and colubrids in general, and estimated neutral substitution rates across 4-fold degenerate sites for the garter snake and other squamates reptiles.

## Materials and Methods

### Genome Sequencing and Annotation

All animal procedures were conducted with registered IACUC protocols (see [supplementary Methods](#)

and sample 4-fold degenerate sites for seven squamates (glass lizard, *Crotaphytus wislizeni*; green anole, *Anolis carolinensis*; bearded dragon, *Basiliscus vittatus*; king cobra, *Ophiophagus hannah*; garter snake, *Thamnophis sirtalis*; cottonmouth, *Agkistrodon cottonmouth*; and Burmese python, *Python bivittatus*) and three mammal outgroups (chimpanzee, *Pan troglodytes*; human, *Homo sapiens*; and mouse, *Mus musculus*); these data were subsampled from the larger 1:1 orthologous gene alignments used for analyses of positive selection in this study (see [supplementary Methods 1.4](#), [Supplementary Material](#) online for details). We estimated branch-specific substitution rates using a relaxed log-normal clock model in BEAST v2.4.7 (Bouckaert et al. 2014) by calibrating the divergence times of the 9 internal nodes (see [supplementary table S4](#), [Supplementary Material](#) online for node calibrations and prior distributions) and constraining the topology to (((GlassLizard, (Anole, Pogona)),((Cobra, Thamnophis), CottonMouth), Python)),((Chimp, Human), Mouse)). Two BEAST analyses were conducted for 100 million generations each, sampling parameter values every 10,000 generations. We discarded the first 25% of runs as burn-in (25 million) and confirmed convergence to the posterior by comparing the likelihood values and effective samples sizes (ESS > 1000 for all parameters).

### Sex Chromosomes

To identify sex chromosome-specific scaffolds in the *T. sirtalis* genome, we used BLAST to locate six gene fragments known to have both Z and W-specific alleles in snakes (Matsubara et al. 2006; Vicoso et al. 2013; Laopichienpong et al. 2017). Maximum likelihood phylogenetic analyses on these gene fragments from several squamates were used to verify sex linkage, which would produce a pattern where Z and W alleles from distinct species cluster together phylogenetically. We also used sex-specific amplification of the *W* gene fragments by PCR to confirm that these scaffolds were sex-linked gametologous alleles and not paralogs or misassemblies. Fluorescent *W* hybridization (FISH) of a *W*-like repeat, (GATA)<sub>n</sub>, was used to identify the W chromosome in the karyotype of a female *T. sirtalis*. See [supplementary Methods 1.5](#), [Supplementary Material](#) online for details.

### Identification and Analysis of microRNA and Associated mRNA Targets

The sequence-specific microRNA prediction (SMIRP) tool was used for the identification of microRNAs that are specific to *T. sirtalis* (Peace et al. 2015), and these predictions were tested in a number of ways, as described in [supplementary Methods 1.6](#), [Supplementary Material](#) online. Inferred mature microRNAs were putatively matched to target coding sequences in the *T. sirtalis* genome using miRanda v.3.3a (Enright et al. 2003), using the parameters described in the [supplementary Methods 1.6](#), [Supplementary Material](#) online.

### Analyses of Visual Gene Loss and Opsin Expression Localization

To identify the full repertoire of visual genes in the garter snake genome, we used BLAST (Altschul et al. 1990) to search the complete genome sequence, as well as the genomes of the king cobra (*Ophiophagus hannah*) and Burmese python (*Python bivittatus*), using a library of visual and opsin genes compiled from previous studies of vertebrate vision (Schott et al. 2017, 2018). Genes that could not be recovered in any of the three snake genomes were presumed to be lost. Gene loss in snakes was compared with losses in mammals, geckos, and crocodylians, three groups with hypothesized nocturnal or dim-light ancestry. Under approved animal protocols at the University of Toronto, we conducted immunohistochemistry to identify the location of opsin gene expression across cells in the *T. sirtalis* retina based on previously published methods (described in Schott et al. 2016; Bhattacharyya et al. 2017). See [supplementary Methods 1.7](#), [Supplementary Material](#) online for additional experimental details.

### Annotation and Phylogenetic Analysis of Olfactory Receptors (ORs)

To infer patterns of expansion and loss in ORs in snakes, we identified and analyzed OR genes from the garter snake and other reptile genomes. OR annotations from the green anole and Burmese python were collected from Vandeweghe et al. (2016).

venom, and compare this to inferences based on RNAseq, we extracted and analyzed crude venom from *T. sirtalis* and *T. elegans* using SDS-PAGE and MALDI-TOF mass spectrometry, under approved animal protocols at the University of Northern Colorado (#9204). See [supplementary Methods 1.9, Supplementary Material](#) online for additional details.

## Analysis of Tetrodotoxin (TTX) Resistance in Voltage-gated Sodium Channels

Garter snakes are well known for their resistance to TTX in their prey, which at the molecular level derives from resistant voltage-gated sodium channels. Because automated annotation approaches performed poorly on these genes, we manually predicted and extracted sequences of all sodium channel alpha subunit ( $\alpha$ ) paralogs from the *T. sirtalis* genome and other published genomes, including three snakes (*B. m. m.*, *B. m. t.*, and *B. m. s.*), two lizards (*A. s. s.* and *A. s. t.*), a bird (*C. c. c.*), a turtle (*C. s. s.*), the human, and as an outgroup, a frog (*X. l. l.*). We focused on the evolution of three voltage-gated sodium channels previously undescribed in *T. sirtalis*: SCN5A ( $\text{Na}_v1.5$ ), SCN10A ( $\text{Na}_v1.8$ ), and SCN11A ( $\text{Na}_v1.9$ ). We aligned these related paralogs using MUSCLE (Edgar 2004), built a gene tree using RAxML (Stamatakis 2014), and performed ancestral sequence reconstruction using the codeml program in PAML v4.8 (Yang 2007).

## Results and Discussion

### Genome Assembly and Annotation

The garter snake represents the first fully annotated genome available for any colubrid snake and is available at NCBI: version *Thamnophis\_sirtalis*-6.0, BioProject PRJNA294278, GenBank accession GCA\_001077635.2. The scaffolded genome assembly is 1.42 Gbp in length (including gaps), with an N50 contig size of 10.4 kb and scaffold N50 size of 647.6 kb. The *T. sirtalis* genome annotation contains ~85% of BUSCO genes, making it more complete than the glass lizard (*A. s. s.*; 84%) and king cobra (*B. m. s.*; 71%) genomes, but less complete than the genomes of the python and other two lizards assessed (*A. s. t.* and *A. s. s.*), which each contained at least 95% of vertebrate BUSCOs ([supplementary fig. S1, Supplementary Material](#) online). Kmer-based estimation of genome size resulted in estimates of 1.19 Gbp and 1.27 Gbp (mean = 1.23 Gbp) using 19mers and 23mers, respectively. These estimates are substantially smaller than the



Material online). Furthermore, the median exon length is very similar in all four species (

chromosome differentiation across lineages (



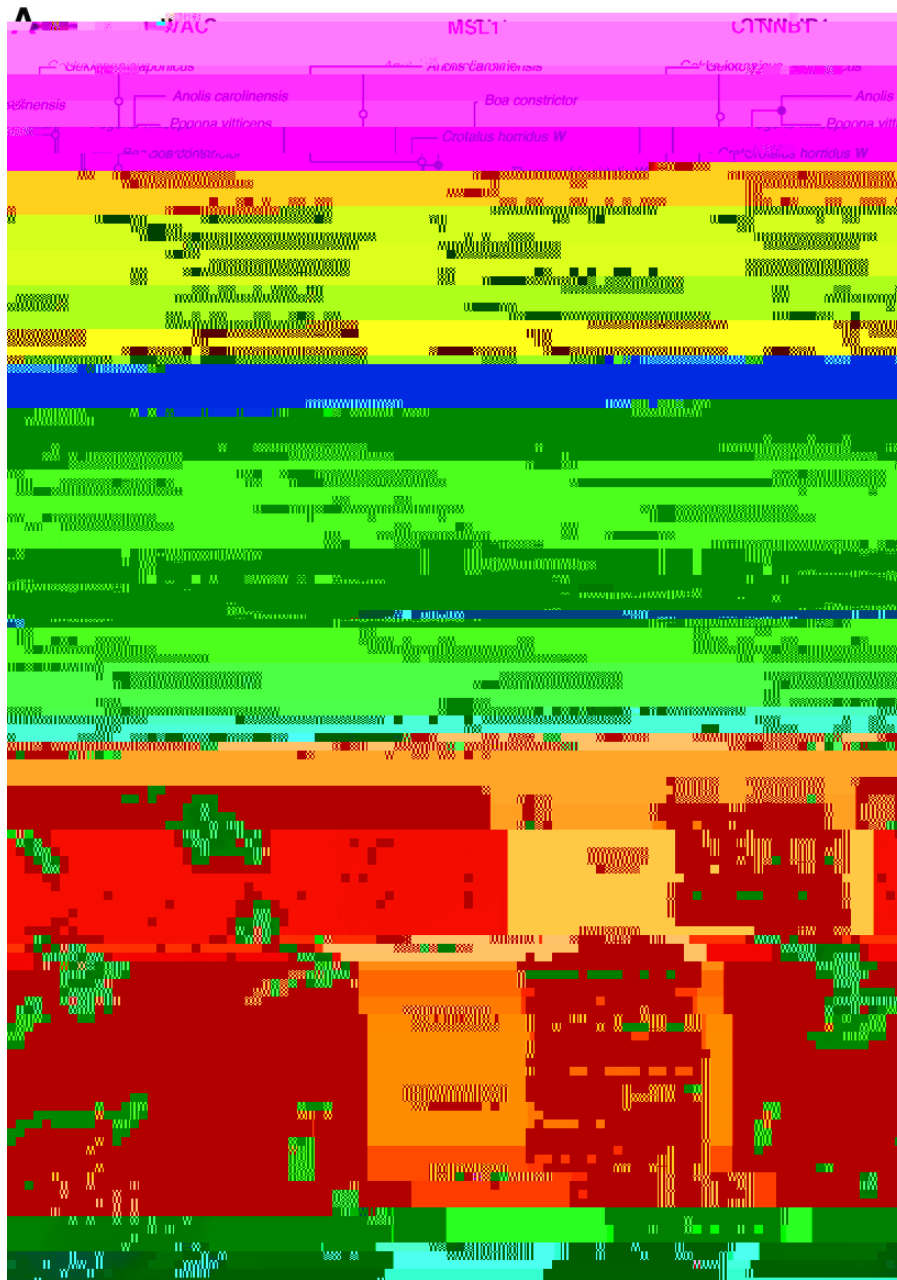


Fig. 3.—Identification of the W chromosome and sex-chromosome-linked genes in six species with Z and W alleles in (4) Maximum likelihood trees of fragments of

(supplementary file S1, Supplementary Material online). Nucleotide frequencies within mature microRNAs were found to be highly similar between the garter snake and king cobra genomes when compared with human, with a high frequency of uracil base at position 9, just outside of the 2–8 seed region responsible for target binding (supplementary fig. S4, Supplementary Material online). A total of 576 mRNA

transcripts were predicted to be targets of the novel mature microRNAs (supplementary file S1, Supplementary Material online). Notably, the novel microRNA, miR-14, was found to target several components of phosphoinositide metabolism; namely, phosphoinositide-3-kinase, regulatory subunit 5 (PIK3R5, also called p101), and inositol polyphosphate-4-phosphatase (INPP4A). Interestingly, PI3K

is responsible for the production of phosphatidylinositol 3, 4, 5-triphosphate (PIP<sub>3</sub>) from the phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PIP<sub>2</sub>). PIP<sub>2</sub> is a key lipid signaling molecule involved in the signaling cascade that is responsible for initiating the venom production cycle in snake secretory cells following the release and depletion of venom (Jansen and Foehring 1983; Kerchove et al. 2008). We speculate that snake-specific microRNAs may play a role in venom production cycle, perhaps by regulating the metabolism of PIP<sub>2</sub>. As such, it is likely that snake-specific microRNAs are involved in specific cellular pathways that contribute to unique facets of its biology.

### Evolution of Genes Related to Vision, Olfaction, and Venom

Several multigene families are of particular interest in snakes due to their association with unique aspects of snake biology. Previous work in the python and cobra has shown significant expansions of gene families in terminal snake branches, including gene families involved in chemoreception (i.e., vomeronasal, olfactory, and ephrin-like receptors) and vision (i.e., opsin genes), which are associated with the utility of the snake tongue and the hypothesis of a fossorial ancestor to all snakes, respectively. Additionally, genes families involved in the production of snake venom proteins are important for

that the apparent all-cone retina of a diurnal garter snake, *Thamnophis sirtalis*, evolved not through loss of the rods, but rather through transmutation (i.e., evolutionary modification) of the rods to resemble the appearance and function of

cones (Schott et al. 2016). This provided the first molecular support for



garter snake genome for cystatin, hyaluronidase, SVSP and

To assess whether these putative toxin genes contribute to the venom secretions of *Coluber constrictor*, we compared their expression levels in the transcriptomes of various tissues, including the venom gland (Duvernoy's gland). There is growing evidence that many venom toxins have evolved from genes that are co-expressed in at least a few different tissue types, and following their recruitment, exhibit significantly higher levels of transcription in the venom gland than other tissues (Vonk et al. 2013; Hargreaves et al. 2014; Junqueira-de-Azevedo et al. 2015; Reyes-Velasco et al. 2015). We compared the expression level of the venom homologs identified in the *C. constrictor* genome in eight different tissues: The venom gland, brain, kidney, liver, lung, ovaries and the lower and upper segments of the digestive tract. Of the 15 genes encoding venom toxins found in the garter snake, 13 were expressed in

The three other members of the voltage-gated sodium channel, Na<sub>v</sub>1.5 (found in cardiac muscle and encoded by the gene *SCN5A*), 1.8, and 1.9 (both found in small sensory neurons and encoded by *SCN10A* and *SCN11A*, respectively) have not been previously described in *Drosophila*. These three channels are closely related, arising from tandem gene duplications before the mammal-reptile split (Dib-Hajj et al. 1999; Zakon et al. 2011; Widmark et al. 2011). In mammals, all three channels display TTX resistance due to the substitution of a non-aromatic residue for an aromatic one in the domain I (DI) P-loop (Backx et al. 1992; Satin et al. 1992; Akopian et al. 1996; Cummins et al. 1999; Leffler et al. 2005). Previously, TTX resistance of these channels was thought to be ubiqui-

Na<sub>v</sub>1.4a (Backx et al. 1992; Satin et al. 1992; Perez-Garcia et al. 1996; Penzotti et al. 1998; Venkatesh et al. 2005). (For each substitution we discuss, the ancestral amino acid refers to mammalian Na<sub>v</sub>1.4, a TTX-sensitive channel, and the position number refers to the location for the specific . . . )



to that in mammals, and generally slightly lower than that of lizards. Identification of Z and W sex chromosome-specific scaffolds in the garter snake genome assembly highlight the value of this genome resource for future studies of sex chromosome evolution. Evolutionary analyses of gene families involved in snake sensory systems reveal patterns of gene loss in visual gene families that strongly support dim-light ancestry in snakes, suggesting that the all-cone retina of *Coluber constrictor* evolved through transmutation of rods into cone-like cells. Additionally, these analyses provide evidence for a major expansion of OR repertoires early in snake evolution, but with no further expansions associated with subsequent diversification or life history shifts in the snake lineage. Finally, we provide one of the first detailed analyses to characterize and link the protein composition of venom, the genes that encode these proteins, and their evolutionary origins in a rear-fanged venomous colubrid species, together with new insight into the evolution of TTX resistance in some garter snakes in response to toxic newt prey. Collectively, our analyses demonstrate a broad spectrum of genomic adaptations linked to the many extreme and unique features of snakes, while also highlighting the variation in genomic features among snakes that together demonstrate why snakes represent intriguing and valuable model systems for diverse research questions.

## Supplementary Material

Supplementary data are available at [Genome Biology](#) online.

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