Proteomic analysis of the mandibular glands from the Chinese crocodile lizard, *Shinisaurus crocodilurus* – Another venomous lizard?

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A B S T R A C T

Based on its phylogenetic relationship to monitor lizards (Varanidae), Gila monsters (Heloderma spp.), and the earless monitor *Lanthanotus borneesis*, the Chinese crocodile lizard, *Shinisaurus crocodilurus*, has been assigned to the Toxicofera clade, which comprises venomous reptiles. However, no data about composition and biological activities of its oral secretion have been reported. In the present study, a proteomic analysis of the mandibular gland of *S. crocodilurus* and, for comparison, of the herbivorous Solomon Island skink *Corucia zebrata*, was performed. Scanning electron microscopy (SEM) of the teeth from *S. crocodilurus* revealed a sharp ridge on the anterior surface, but no grooves, whereas those of *C. zebrata* possess a flattened crown with a pointed cusp. Proteomic analysis of their gland extracts provided no evidence of venom-derived peptides or proteins, strongly supporting the non-venomous character of these lizards. Data are available via ProteomeXchange with identifier PXD039424.

1. Introduction

Animal venoms evolved in a wide phylogenetic range of organisms for predatory and defensive purposes (Fry et al., 2009a; Calvete 2017; Schendel et al., 2019; Zancolli and Casewell, 2020). In reptiles, venom has been hypothesized to originated just once, approximately in the mid-Jurassic (170 mya), in a monophyletic clade coined Toxicofera that combines the suborders Serpentes, Anguimorpha, and Iguania of scaled reptiles (Vidal and Hedges, 2009; Fry et al., 2006, 2012; Douglas et al., 2010; Koludarov et al., 2017).

Toxicofera includes *Heloderma* species, which are well known to produce a toxic secretion, i.e. venom, in the submandibular gland. In other anguimorphan lizards, like the *Varanidae* species, both the existence of a "venom system" and their denomination as "venomous" animals have been called in question (Weinstein et al., 2012, 2013; Kardong 2012; Sweet, 2016). This also applies to the earless monitor lizard *Lanthanotus borneensis* (Mebs et al., 2021), a close relative of the Varanidae family (Ast 2001; Douglas et al., 2010; Wiens et al., 2012; Pyron et al., 2013). Proteomic analysis confirmed the presence of kallikrein as

the major biologically active component of the monitor lizard's submandibular gland extract (Mebs et al., 2021), as shown also in previous studies (Koludarov et al., 2017; Fry et al., 2006, 2009b, 2010a, 2010b). However, in view of the *L. borneensis*'s biology, this enzyme, which has been considered to be an important constituent of anguimorphan "venoms", does not appear to play an active part in prey acquisition or defence (Mebs et al., 2021).

First collected in 1928 (Ahl, 1930; Fan, 1931), the Chinese crocodile lizard, *Shinisaurus crocodilurus* (Fig. 1A), represents a monotypic genus of the family Shinisauridae (Hu et al., 1984). Its species name refers to the crocodile-like tail- and back-scalation. Phylogenetic studies indicate a close relationship of *Shinisaurus* to monitor lizards (*Varanus* spp.), *Heloderma* spp., and in particular to *Lanthanotus borneensis* (Townsend et al., 2004; Li et al., 2012; Pyron et al., 2013). *Shinisaurus crocodilurus* is a viviparous, diurnal, semiaquatic lizard and a strong swimmer, with habitat restricted to specific areas of mountainous locations in Southeast-China and Northeast-Vietnam, where it lives in shallow waters near riverbanks preying on worms, aquatic insects and their larvae, snails, tadpoles, and fish (Zhao et al., 1999Zhao et al., 1999). An
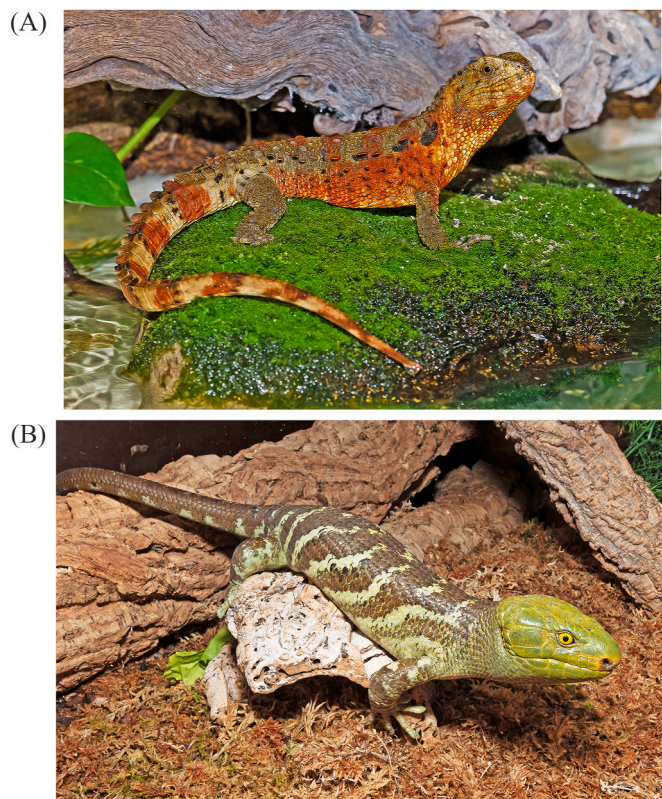

Fig. 1. The Chinese crocodile lizard *Shinisaurus crocodilurus* (**A**) (Photo: G. Stelzner) and the Solomon Island skink *Corucia zebrata* (**B**) (Photo: M. Zollweg).

integrative (morphological, molecular, and ecological) taxonomic approach revealed that the northeastern (Chinese) and southwestern (Vietnamese) *S. crocodilurus* represent distinct conservation and taxonomic lineages within this ancient (Early Paleocene, 59,7 mya) and evolutionarily conserved clade (Van Schingen et al., 2016). Phylogenetic analyses strongly support the presence of four genetically distinct populations, three from China and one from Vietnam (Huang et al., 2014; Nguyen et al., 2022b).

Both males and females crocodile lizards exhibit extensive colour variation, particularly in the throat and ventral regions (Qiu et al., 2022), turning this reptile into a prized pet. The Chinese crocodile lizard has been successfully bred in captivity (Kudrjawtsew and Wasiljew, 1991; Zollweg and Kühne, 2013). However, this has not prevented it from being listed as a critically endangered CITES Appendix I species since 2014 (Nguyen et al., 2022a). Anthropogenic threats, including habitat loss and degradation and overcollection for the international pet trade, are sharply declining the population sizes of crocodile lizards throughout their range (Nguyen et al., 2022b). A 2004 survey estimated the wild Chinese population in 950 individuals (Huang et al., 2008), while the size of the Vietnamese population was less than 200 individuals in 2016 (Van Schingen et al., 2016).

Although no data exist regarding the biological activity of its oral secretions, the taxonomic position of *S. crocodilurus* within the suborder Anguimorpha of a proposed Toxicofera clade that includes all venomous reptiles (Fry et al., 2010a, 2012) would suggest that this species might represent a hitherto unknown member of the group of venomous reptiles. In the present study, a proteomic analysis of the submandibular glands of *S. crocodilurus* was performed to find out, whether this organ produces a putative venom. For comparison, the homologous mandibular glands of a non-Toxicofera, strictly herbivorous lizard, the Solomon Island skink *Corucia zebrata* (Fig. 1b), was analysed.

2. Materials and methods

The right and left mandibular glands were excised from single captive bred specimens of *S. crocodilurus* (adult male, 38 cm total body length) and the Solomon Island skink, *C. zebrata* (adult male, 35 cm total body length), both of which had been kept frozen at −20 °C for about six months after their natural death. The glands were placed in separate 5 ml Eppendorf ProteinLoBind tubes and homogenized in physiological saline at 4 °C by ultrasonication. After centrifugation at 3.000 rpm and 4 °C for 15 min, the supernatants were immediately lyophilized and submitted to proteomic analysis.

2.1. Scanning electron microscopy (SEM)

The lower jaw of the lizards was mounted on an aluminium holder, sputtered with gold, and analysed with a Hitachi S-4500 scanning electron microscope at an acceleration voltage of 5 kV (cold-field emission electron source).

2.2. Proteomic analyses

The mandibular gland extracts of *S. crocodilurus* and *C. zebrata* were analysed using two proteomic strategies: a 'shotgun' MS/MS approach (Lomonte et al., 2020) at the Proteomics Unit of Instituto Clodomiro Picado, and an SDS-PAGE decomplexation bottom-up LC-MS/MS workflow (Calvete, 2014) at proteomics facility of Instituto de Biomedicina de Valencia.

For the shotgun analysis, 15 μg of total proteins extracted from the pooled left and right gland extract were diluted in 25 mM ammonium bicarbonate and subjected to reduction with 10 mM dithiothreitol (30 min at 56 °C), alkylation with 50 mM iodoacetamide (20 min in the dark), and digestion with sequencing grade trypsin (0.25 μg/sample, 37 °C, overnight). After stopping the reaction with 0.5 μL of formic acid, the tryptic peptide digests were centrifuged and separated by RP-HPLC on a nano-Easy 1200 chromatograph (Thermo) on-line with a Q-Exactive Plus® mass spectrometer (Thermo). Ten μL samples (~0.7 μg of peptide mixture) were loaded onto a C18 trap column (75 μm × 2 cm, 3 μm particle; Thermo), washed with 0.1% formic acid (solution A), and separated at 200 nL/min on a C18 Easyspray PepMap® column (75 μm × 15 cm, 3 μm particle; Thermo). A gradient toward solution B (80% acetonitrile, 0.1% formic acid) was developed for a total of 45 min (1–5% B in 1 min, 5–26% B in 30 min, 26–79% B in 6 min, 79–99% B in 2 min, and 99% B for 6 min). MS spectra were acquired in positive mode at 1.9 kV, with a capillary temperature of 200 °C, using 1 μscan in the range 400–1600 m/z, maximum injection time of 50 msec, AGC target of 1×10^6, and resolution of 70,000. The top 10 ions with 2–5 positive charges were fragmented with AGC target of 3×10^6, minimum AGC 2×10^3, maximum injection time 110 msec, dynamic exclusion time 5 s, and resolution 17,500. MS/MS spectra were processed against protein sequences contained in the UniProt database for Lepidosauria (December 2022; 624,826 entries) using Peaks X® (Bioinformatics Solutions). Parent and fragment mass error tolerances were set at 15.0 ppm and 0.5 Da, respectively. Cysteine carbamidomethylation was set as fixed modification, while methionine oxidation and deamidation of asparagine or glutamine were set as variable modifications. A maximum of 2 missed cleavages by trypsin in semispecific mode were allowed. Filtration parameters for match acceptance were set to FDR<0.1%, detection of ≥1 unique peptide, and −10lgP protein score ≥30.

For the bottom-up strategy, the proteins extracted from the left and right mandibular gland of *S. crocodilurus* and *C. zebrata* were separated by SDS-PAGE in 10% polyacrylamide gels run under reduced conditions. Protein bands were excised from Coomassie Brilliant Blue-stained gels and subjected to automated in-gel reduction and alkylation (as described above for the 'shotgun' MS/MS approach) on a Genomics Solution ProGest™ Protein Digestion Workstation. Tryptic digests were submitted to MS/MS analysis on a nano-Acquity UltraPerformance LC®
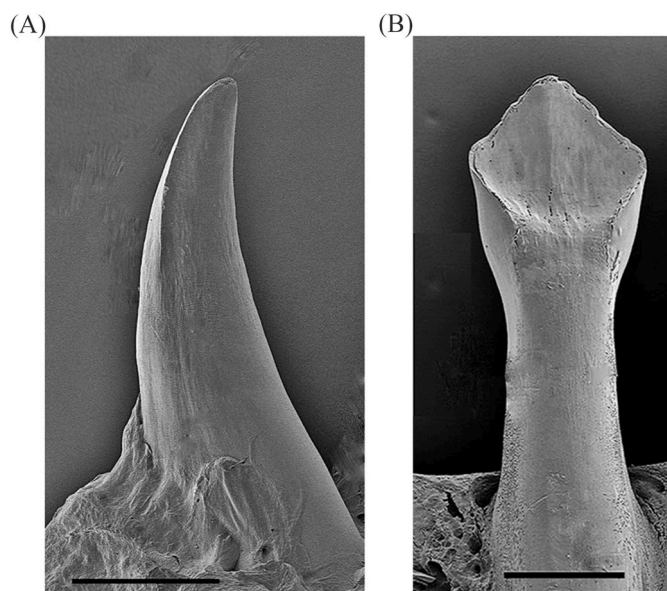
Fig. 2. Scanning electron microscopy of mandibulary pleurodont teeth of *Shinisaurus crocodilurus* (**A**) and *Corucia zebrata* (**B**). Bar – 1 mm.

(UPLC®) equipped with a BEH130 C$_{18}$ (100 μm × 100 mm, 1.7 μm particle size) column in-line with a Waters SYNAPT G2 High Definition mass spectrometer. Doubly and triply charged ions were selected for CID-MS/MS. Fragmentation spectra were submitted to MASCOT Server (version 2.6) at http://www.matrixscience.com and matched against the [bony vertebrates] taxonomy restricted dataset of the NCBI non-redundant database (release 252 of October 15, 2022). Search parameters were: enzyme: trypsin (two-missed cleavage allowed); MS/MS mass tolerance for monoisotopic ions: ±0.6 Da; carbamidomethyl cysteine and oxidation of methionine were selected as fixed and variable modifications, respectively. Assignments with significance protein score threshold of p < 0.05 (Mascot Score >43) were taken into consideration, and all the associated peptide ions hits were manually validated.

2.3. Data accesibility

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Perez-Riverol et al., 2022) under Project Name: Proteomic analysis of the mandibular glands from the Chinese crocodile lizard, Shinisaurus crocodilurus – another venomous lizard? and dataset identifier PXD039424.

3. Results and discussion

3.1. Scanning electron microscopy of teeth

The mandibular pleurodont teeth of *S. crocodilurus* are recurved, not grooved, or serrated but are characterized by exhibiting a sharp, razor-like ridge on the anterior surface (Fig. 2A). Bites of the lizard are inflicting bleeding wounds, but no local or systemic envenoming symptoms occur (Zollweg, personal observation). In contrast, the teeth of *C. zebrata* are entirely different (Fig. 2B). They possess a symmetrical flattened crown tipped by a pointed cusp (Mahler and Kearny, 2006; Hutchinson and Scanlon 2009). These morphologies depart from the grooved or hollow teeth convergently evolved in both rear- or front-fanged venomous Caenophidian snakes to deliver venom (Vonk et al., 2008; Kerkkamp et al., 2015). The seemingly absence of venom delivery structures makes it improbable that both, the Chinese crocodile lizard, *S. crocodilurus*, and the Solomon Island skink, *C. zebrata*, possess venom delivery systems.

3.2. Proteomic analyses of gland extracts

Results of the shotgun proteomic analysis of the *S. crocodilurus* and *C. zebrata* mandibular gland extracts are summarized in Supplemental Tables S1 and S2, respectively. The outcome of the gel-based bottom-up LC-MS/MS assignments of the proteins extracted from the left and the right mandibular glands are displayed in Table S3. Both proteomic workflows complement each other and together converged in identifying a number of proteins from lepidosaurian taxa, both lizards and snakes. 621 and 539 hits were identified, respectively, in the shotgun analysis of *Shinisaurius* and *Corucia* gland extracts, and 59 entries were assigned through bottom-up LC-MS/MS in each gland extract. None of these hits corresponded to any reported putative anguimorphan toxin gathered from i) proteomic analysis of *Lanthanotus borneensis* (Mebs et al., 2021); ii) transcriptomic analysis of the mandibular gland of *Heloderma horridum* (beaded lizard) (Lino-López et al., 2020) and protein sequencing of its protein secretion (Nikai et al., 1988; Utaisincharoen et al., 1993); iii) transcriptomic analysis of the mandibular gland of a number of helodermatid and varanid taxa (Fry et al., 2006, 2009b; Koludarov et al., 2014, 2017); and iv) the genome sequence of *Varanus komodoensis* (Lind et al., 2019). Furthermore, no single toxin-derived peptide homologous to those representing biosynthetic products of snake venom glands (Zancolli and Casewell, 2020) were recovered in the proteomic datasets of *S. crocodilurus* and *C. zebrata* mandibular gland extracts displayed in Tables S1–S3. Supplemental Table S4 lists the 29 and 69 entries retrieved from the NCBI database using, respectively, the search details ("Varanidae" [Organism] OR Varanidae [All Fields]) AND toxins [All Fields] snd ("Heloderma" [Organism] OR Heloderma [All Fields]) AND toxins [All Fields]. These entries include homologues of toxins that may potentially induce hypotension (kallikrein, B-type natriuretic peptide, helokinestatin), inhibit blood coagulation (type III PLA$_2$), trigger unregulated activation of the complement cascade (CVF, cobra venom factor), cause painful cramping and hyperalgesia (AVIToxin), and impair smooth muscle physiology (CRISP, exendin, helofensin) (Fry et al., 2009b; Koludarov et al., 2014). On the contrary, all the identified *Shinisaurius* and *Corucia* mandibular gland proteins belong to different structural classes of housekeeping molecules, mainly cytoskeletal (lamin, actin and actin-binding proteins, such as tropomyosin, moesin, coronin, profilin, gelsolin, plexin); proteins that form part of the framework of the extracellular matrix anchoring basement membranes to the underlying connective tissue (collagens, dermatopontin, prolargin, decorin); and constituents of intermediate filaments (vimentin) and other macromolecular systems, e.g. myosin (muscle), keratin (skin), ribosomal proteins (ribosomes), proteasome subunits. Other proteins are classified as ligand binders and/or transporters (serum albumin, transferrin, hemoglobin, calreticulin, annexin, hemopexin, galectin); regulatory molecules (14-3-3 protein zeta-like protein, histones, heat shock proteins, 78 kDa glucose-regulated protein, endoplasmin, peroxiredoxin, prothymosin, osteoglycin, translation initiation and elongation factors); ubiquitous enzymes (L-lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, aldehyde dehydrogenase, superoxide dismutase, triosephosphate isomerase, protein disulfide-isomerase, cytochrome *c* oxidase, aspartate aminotransferase, cathepsin B, ATP synthase, malate dehydrogenase, enolase, creatine kinase, galactose-3-O-sulfotransferase, glutathione S-transferase). These findings appear to be incompatible with the hypothesis that the mandibular gland tissues of both lizards, *S. crocodilurus* and *C. zebrata*, here studied, actually belong to a venom-producing gland.

4. Concluding remarks

Based on its many primitive anatomical features, *S. crocodilurus* is considered a remnant reptile from the Pleistocene (Huang et al., 2008), thus bearing a great taxonomic value for understanding the origin and evolutionary radiation of Toxicofera squamates and their venoms. The
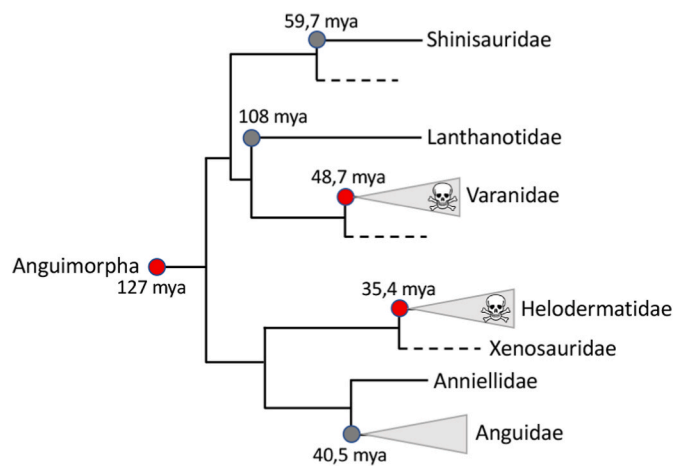
Fig. 3. Scheme of phylogenetic relationships of squamate reptiles of the Anguimorpha suborder (adapted from Douglas et al., 2010; Reeder et al., 2015). Estimated mean divergence times for selected nodes are indicated in millions of years (mya). The most recent common ancestor (MRCA) of the Anguimorpha clade has been dated in the Early Cretaceous, 127.1 (105.5–148.7) mya; the MRCA node for Lanthanotidae has been estimated to be Mid Cretaceous (108.3 (83.3–133.2) mya); divergence time of 59.7 (17.6–100.3) million years (Early Paleocene) has been inferred for Shinisauridae. The MRCA for Varanidae was placed in the Early Eocene (48.7 (30.7–73.6) mya), and the divergence of Helodermatidae has been dated at 35.4 (29.4–41.4) mya. Clades including documented venomous taxa are labeled with the skull and crossbones symbol.

extant monophyletic Anguimorpha clade is split into two groups (Fig. 3) (Douglas et al., 2010; Reeder et al., 2015; Brennan et al., 2021). One clade includes the lineages Shinisauridae and Lanthanotidae, represented respectively by the non-venomous Chinese crocodile lizard *Shinisaurus crocodilurus* (this work) and the earless monitor lizard *Lanthanotus borneensis* (Mebs et al., 2021) as sister taxa to Varanidae (which among its roughly 80 described members includes venomous varanid species, such as the Komodo dragon, *Varanus komodoensis* (Brennan et al., 2021). The second group includes lineages (Anguidae, Xenosauridae, Anniellidae) where no evidence of venomous species has been reported to date, and Helodermatidae, represented by the North American helodermatid lizards included genus *Heloderma* (Douglas et al., 2010), i.e. the Gila monsters *H. horridus* and *H. suspectus* known to produce venom. Comparative transcriptomics of venom and salivary glands and other body tissues within and between venomous and non-venomous snake species has casted doubts on the single, early evolution of the reptile venom system (Hargreaves et al., 2014; Reyes-Velasco et al., 2015). However, clearly, settling this issue requires a comparative genomics approach. Venom is a metabolically expensive secretion and a relaxation of positive pressure acting upon the venom, eg, in response to dietary adaptation towards a prey, such as fish egg, whose capture does not require venom, may favour secondary atrophy of the venom production system (Li et al., 2005a; 2005b). Our work revealing the lack of morphological (hollow teeth) and biochemical (glandular toxins) traits expected for a venomous animal indicates that labelling the extant Chinese crocodile lizard *Shinisaurius crocodilurus* as venomous animals is not justified. Whether this is the result of atrophy of an ancestral venom-producing system or, conversely, that the Shinisaurus evolutionary line lacked this system, requires further studies. Genomes encode traces of events from the evolutionary history of the species. Deep analysis of the genome of *Shinisaurus crocodilurus* (Gao et al., 2017) may contribute to the elucidation of the biology of this organism, and inter- and intra-specific comparative genomics will uncover a treasure trove of biological information to reconstruct the molecular bases of the evolution of venom genes across Toxicofera.

Author contributions

Conceptualization, DM; Methodology, BL, MZ, JJC; Formal analysis, BL, MZ, CL, DP; Data curation, BL, JJC; writing—original draft preparation, DM; writing—review and editing, DM, BL, MZ, JJC, DP, CL; Funding acquisition, JJC. All authors have read and agreed to the final version of the manuscript.

Ethical statement

Authors declare that international ethical guidelines for scientific papers were followed in the preparation of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are available via ProteomeXchange with identifier PXD039424

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2023.107050.

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