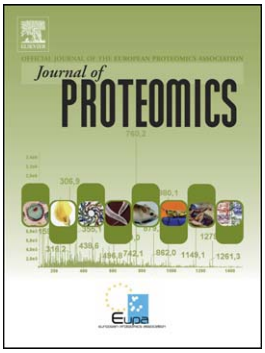

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A ray of venom: combined proteomic and transcriptomic investigation of fish venom composition using barb tissue from the blue-spotted stingray (*Neotrygon kuhlii*)

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ABSTRACT

Fish venoms remain almost completely unstudied despite the large number of species. In part this is due to the inherent nature of fish venoms, in that they are highly sensitive to heat, pH, lyophilisation, storage and repeated freeze-thawing. They are also heavily contaminated with mucus, which makes proteomic study difficult. Here we describe a novel protein-handling protocol to remove mucus contamination, utilising ammonium sulfate and acetone precipitation. We validated this approach using barb venom gland tissue protein extract from the blue-spotted stingray *Neotrygon kuhlii*. We analysed the protein extract using 1D and 2D gels with LC-MS/MS sequencing. Protein annotation was underpinned by a venom gland transcriptome. The composition of our *N. kuhlii* venom sample revealed a variety of protein types that are completely novel to animal venom systems. Notably, none of the detected proteins exhibited similarity to the few toxin components previously characterised from fish venoms, including those found in other stingrays. Putative venom toxins identified here included cystatin, peroxiredoxin and galectin. Our study represents the first combined survey of gene and protein composition from the venom apparatus of any fish and our novel protein handling method will aid the future characterisation of toxins from other unstudied venomous fish lineages.

INTRODUCTION

Venom systems are important ecological innovations that have evolved independently on many occasions throughout the animal kingdom [1,2]. Venoms are bioactive

secretions that are utilised for a variety of functions, such as defense, competitor deterrence or predation [1,2]. Within vertebrates, reptiles and fishes represent the vast majority of all venomous species [3–6]. In contrast to the considerable research effort undertaken to elucidate the composition of venoms from reptiles, in particular snakes due to their medical importance [7], little is known about the composition of venoms present in bony and cartilaginous fishes. In contrast to snakes, fishes appear to primarily use their venom systems to protect themselves from predation. Interestingly, this defensive venom system appears to have evolved independently on many occasions in different cartilaginous (sharks, chimaeriformes and stingrays) and bony fishes (catfish and teleosts) lineages [3–5]. The majority of venomous fish species use spines to inject venom in to potential predators - these spines are typically located dorsally, although a number of species have pectoral, caudal or clitheral venom apparatuses [3,4].

Fish venoms are inherently difficult to study - they have been shown to be highly labile and sensitive to heat, pH, lyophilisation, storage and repeated freezing and thawing [8]. Furthermore, fish venom samples are typically mucus rich, causing undesirable issues during protein separation steps typically applied during proteomic methodologies. The collection of venom itself can also be problematic because the ‘venom gland’ is typically not a well-defined storage structure, instead often simply a grouping of secretory cells present within/along a groove in a spine.

Despite these difficulties, some fish venoms have been partially characterised pharmacologically and a small number of fish venom toxins have been purified and their toxic activities characterised [8,9]. Whilst fish venoms are not typically

considered to be capable of causing mortality, the incidence of envenomings may be high, and such events can result in significant clinical sequelae in envenomed victims, such as paralysis, erythema, itching, and persistent local pain disproportionate to the wound size [4,8–10]. Consistent with the hypothesis that defensive venoms likely evolve slowly due to having a conserved biological activity, fish venoms have been found to produce broadly similar pathologies despite their apparent independent evolution [4,9]. Broadly speaking, the biological activity of, and the resulting pathology induced by, fish venoms appear to be intrinsically linked to the presence of cytolytic components [9], with extreme local pain being the dominant feature of envenomations.

Despite the relative medical importance of fish venoms and the pharmacological interest in their toxins, as described above, their toxic composition remain almost completely uncharacterised at the molecular level. At the time of writing there are only 21 fish toxin sequences in the UniProt database (http://www.uniprot.org) and only one transcriptomic study of a fish venom gland has been published [11]. The absence of basic proteomic studies on venomous fishes is particularly surprising, yet only a few recent studies have attempted to investigate this subject [12,13].

Here we address this knowledge deficiency by characterizing the composition of venom from the blue-spotted stingray (*Neotrygon kuhlii*). Stingrays (Chontrichthyes: Myliobatoidei) are a group of cartilaginous fishes that are commonly found occupying the demersal zone of tropical and subtropical marine waters, although some species are found in freshwater and pelagic environments. Most rays have retroserrate spines located on their caudal appendage (Figure 1) – the spines are layered by dermis,

venom glands and epidermis and then encapsulated by an integumentary sheath, resulting in a structure termed a 'barb' [3]. This venom system is used defensively to protect the animal from predation and potential aggressors.

Whilst the venoms of most stingrays remain completely unstudied, the venom activities of the South American freshwater stingrays of the family Potamotrgonidae have been partially characterised. *Potamotrygon* venoms induce oedematogenic and nociceptive responses in mice, are capable of causing necrosis and exhibit some proteolytic and hyaluronidase activity [14,15]. Two small peptides, named porflan and orpotrin, have been isolated and characterized from *Potamotrygon* venom and they have been demonstrated to (i) interfere with membrane phospholipids through an as yet uncharacterized mechanism that results in pro-inflammatory activity and (ii) act on large arterioles of the microcirculatory network resulting in vasoconstriction, respectively [16,17].

In contrast to *Potamotrygon* stingrays, the blue-spotted stingray (*Neotrygon kuhlii*) (Figure 1) inhabits marine environments, and is found in the tropical and sub-tropical waters of the Indo-Pacific oceans. Here we apply a combined transcriptomic and proteomic approach to characterise the composition of venom extracts collected from the barb of this species. Our study represents the first combined survey of gene and protein composition from the venom apparatus of any fish and our analyses reveal a variety of proteins that are completely novel to animal venom systems. We also describe a novel protein handling method that will be of great value to other researchers interested in characterising the protein composition of toxins found in problematic fish venom samples.

METHODS

Specimens

Neotrygon kuhlii specimens were collected in Moreton Bay, Queensland, Australia under collection permit QS2013/MAN143 and animal ethics approval SBS/345/12/ARC. Spine samples were collected and immediately snap frozen in liquid nitrogen before storage at -80°C until use. A total of five spines were used for proteomic analyses and five for transcriptomics. In total this amounted to spines sampled from 10 specimens.

Transcriptomics

Stingray spines were scraped to collect venom secretory material, which was then homogenized and the RNA extracted using the TRIzol Plus RNA Purification Kit (Life Technologies). RNA quality was assessed using a Bioanalyser (Agilent) and ribosomal RNA removed using the Ribo-Zero rRNA Removal Kit for Human/Mouse/Rat (epicentre). The RNA-Seq library was prepared from 50 ng of the enriched RNA material using the ScriptSeq v2 RNA-Seq Library Preparation Kit (epicentre), following 12 cycles of amplification. The sequencing library was purified using AMPure XP beads (Agencourt), quantified using the Qubit dsDNA HS Assay Kit (Life Technologies) and the size distribution assessed using a Bioanalyser (Agilent). The library was sequenced on a single lane of an Illumina MiSeq machine housed at the Centre for Genomic Research, Liverpool, UK, generating ~3 million paired-end 250 bp reads. The ensuring read data was quality processed, first by

removing the presence of any adapter sequences using Cutadapt [18] and then by trimming low quality bases using Sickle (https://github.com/najoshi/sickle). Reads were trimmed if bases at the 3' end matched the adapter sequence for 3 bp or more, and further trimmed with a minimum window quality score of 20. After trimming, reads shorter than 10 bp were removed. The sequence data was assembled using the *de novo* assembly algorithm in CLC Genomics Workbench v4.9 (CLCBio), with the paired-end read criterion and constraining the contig size to >350 bp in length. The assembled contigs were subsequently annotated with Blast2GO using standard parameters [19]. The sequence data has been submitted to the sequence read archive (SRA) database of GenBank (http://www.ncbi.nlm.nih.gov/sra) with the BioProject number PRJNA240112 and the SRA accession number SRX481088. Nucleotides are available in Supplementary file 1.

Protein Extraction

The proteins from stingray spine scrapings were extracted and cleaned using a novel protein extraction and cleanup method. A solution was prepared on ice using 3.7g EDTA, 5mL 200mM PMSF, 10mL Triton X-100, 1L purified water. The solution was poured over the spines and placed on a magnetic stirrer overnight (>12hr) at 4°C. The solution was then centrifuged at 4,500 RCF, 4°C for 30 minutes, before 80% ammonium sulfate saturation (~43% w/v) was added and the solution placed on a magnetic stirrer at 4°C and left overnight (>12hr). The protein-containing precipitate was then centrifuged at 4,500 RCF, 4°C for 30 minutes. The supernatant was removed and the protein precipitate brought up in purified water (ratio of 15 parts water to 1 part supernatant), vortexed for 2 minutes followed by centrifugation at 14,000 RCF, 4°C for 30 minutes. Subsequently the supernatant was diluted 1:9 with cold 1:4

acetone:methanol. The solution was placed at -20°C and allowed to precipitate overnight (>12hr). The solution was subsequently centrifuged at 14,000 RCF, 4°C for 30 minutes and the supernatant was discarded. The pellet was left to evaporate at room temperature for 1hr, then resolubilised in purified water. The total protein concentration was then measured using a Thermo Scientific Nanodrop 2000 Spectrophotometer in A280 mode. Extracted venom proteins were stored at -80°C.

Proteomics

One Dimensional (1D) SDS-PAGE

Samples were first analysed using 1D SDS-PAGE as previously described [20]. 60 µg of lyophilised venom protein extraction was reconstituted in purified water and 6 µL of standard loading dye buffer (150 mM Tris HCl pH 6.8, 300 mM DTT, 6% SDS, 0.3% Bromophenol Blue, 30% Glycerol). Venom proteins were reduced by boiling the sample at 100°C for 4 minutes. SDS-PAGE gels, composed of a 12% acrylamide resolving gel (3.3 mL purified water, 2.5 mL resolving buffer [1.5M Tris, pH8.8], 4 mL 30% Acrylamide, 100 µL 10% SDS, 100 µL 10% APS, 4 µL TEMED) and a 5% acrylamide stacking gel (1.4 mL purified water, 250 µL stacking buffer [0.5M Tris, pH 6.8], 330 µL 30% Acrylamide, 20 µL 10% SDS, 20 µL 10% APS, 2 µL TEMED), were polymerized and submerged in running buffer (2.4 g Tris, 11.52 g Glycine, 0.8 g SDS, 800 mL purified water). The venom protein extraction sample and 5 µL protein ladder (Precision Plus Protein All Blue Standards, Bio-Rad) were loaded and an electrical current of 120 V applied for 10 minutes and then increased to 140 V for 60 minutes. The gel was removed and stained with Colloidal Coomassie Brilliant Blue G250 (34% MeOH, 3% Phosphoric Acid, 170 g/L Ammonium Sulfate, 1 g/L Coomassie Blue G250) on a shaker overnight, then destained with purified water.

Using protocols previously described [21–23] in-gel digestion was undertaken to extract proteolytic peptides for mass spectrometry analysis. Bands were cut from the gel and destained with 500 µL 50% acetonitrile (ACN), 50 mM ammonium bicarbonate (ABC) - this process was repeated twice. Proteins were reduced using 40 µL 10 mM dithiothreitol (DTT) for 30 minutes at 60°C, then alkylated with 40 µL of 55 mM iodoacetamide (IAA) for 30 minutes in the dark. The protein bands were rinsed twice with 50 mM ABC, then dehydrated with 100 µL 100% ACN and rehydrated with 8 µL of trypsin solution (Sigma, USA) (10 ng/µL in 50 mM ABC) for 10 minutes at 4°C. 16 µL of 50 mM ABC was added and then incubated at 37°C for 16 hours. The solution was then transferred to a new 1.5 mL eppendorf tube (Tube 2) and 50 µL of 50% ACN / 1% formic Acid (FA) added to Tube 1 and the contents vortexed. This solution was transferred to Tube 2, and the previous step repeated twice. The solutions in Tube 2 were lyophilised, then reconstituted in 20 µL 5% ACN / 1% FA in polypropylene inserts in glass sample vials for sequencing using an AB SCIEX 5600 Triple TOF mass spectrometer machine (AB SCIEX, USA). Samples were first desalted on a 0.5 x 3 mm C18 trap (Agilent) for 3 min at a flow rate of 30 µl/min using buffer A (1% ACN / 0.1 % FA), followed by separation on a Vydac Everest C18 column (5 µm, 300 Å, 150 mm x 150 um) at a flow rate of 1 µl/min using a Shimadzu Prominence nanoLC system using a gradient of 10% - 60% buffer B (0.1% FA in CAN) over 30 min (10-60% buffer B over 14 min for samples from 2D gel spots). Eluted peptides were directly analysed on a TripleTof 5600 instrument using a Nanospray III interface. TOF MS scans were performed across m/z 350 – 1800 (0.5 s) followed by data-dependent acquisition of up to 20 peptides across m/z 40 – 1800 (0.05 s) with intensity above 100 counts using a collision energy spread of

40 +/- 15 V. Gas 1 was set to 10 psi, curtain gas to 30 psi, and ion spray floating voltage set to 2700 V. Spectra were analyzed with Proteinpilot V4.0 (ABSciex, USA), then protein/peptides were identified via a UniProt Blast search and searches against the translated assembled transcriptome database.

Two Dimensional Gel Electrophoresis (2DE)

The *N. kuhlii* venom extract (300 µg) was reconstituted with 150 µL of rehydration buffer (8 M Urea, 100 mM DTT, 4% CHAPS, 0.5% ampholytes (Biolytes pH 3-10), 0.01% Bromophenol Blue) and loaded onto a 7 cm IPG strip with a non-linear pH gradient of 3-10 overnight (8+ hours). Using the preset methods (Step 1: 100 volts, rapid gradient, 50 µAmps, 100 volt hours; Step 2: 500 volts, rapid gradient, 50 µAmps, 500 volt hours; Step 3: 1,000 volts, rapid gradient, 50 µAmps, 1,000 volt-hours; Step 4: 4,000 volts, rapid gradient, 50 µAmps, 14,800 volt hours) the IPG strips were isoelectrically focused for a total of 98,400 Vhrs, then stored at -20°C until needed. The IPG strip was equilibrated using 5 mL of reducing buffer (150 mg DTT, 10mL equilibration buffer [36 g Urea, 4mL 50% Glycerol], 6.25 mL gel buffer [1.5 M Tris HCl pH 8.8], 2.5 mL 20% SDS, topped up to 10 mL with purified water) for 10 minutes, then 5 mL alkylating buffer (200 mg IAA, 10 mL equilibration buffer) for 20 minutes. The IPG strip and protein ladder (Precision Plus Protein All Blue Standards, Bio-Rad) were embedded on the top of a 12% SDS-PAGE gel (7 cm x 7 cm x 1 mm) (3.3 mL purified water, 2.5 mL resolving buffer [1.5M Tris, pH8.8], 4 mL 30% Acrylamide, 100 µL 10% SDS, 100 µL 10% APS, 4 µL TEMED) with agarose (0.5% agarose in gel overlay buffer [0.375 M Tris HCl pH 8.8, 0.1% SDS]). The gel cast was submerged with electrophoresis running buffer (12 g Tris, 57.6 g Glycine, 4 g SDS, 800 mL purified water) and an electrical current of 20 mA applied

for 10 minutes and then 60 mA for 1 hour. The resulting gels were stained using 0.2% Colloidal Coomassie Brilliant Blue G250 for approximately two days, then de-stained with purified water for up to one week.

Using protocols previously described [21–23] in-gel digestion was undertaken to extract and cleave proteins for mass spectrometry analyses. Spots were picked and placed in PCR tubes (Tube 1). They were washed with 100 µL purified water for 15 minutes, then destained twice with 150 µL 40 mM ammonium carbonate (AC) / 50% ACN for 30 minutes. Spots were dehydrated using 200 µL ACN for 10 minutes, then digested overnight (8+ hours) with 10 µL of trypsin solution (10 ng/µL in 50 mM ABC) at 37°C. The trypsin solution was transferred from Tube 1 to a 1.5 mL eppendorf tubes (Tube 2). The gel spot in tube 1 was washed using 20 µL 1% FA for 20 minutes, then the solution was transferred to Tube 2. The gel spot in tube 1 was washed with 20 µL of 5% ACN / 1% FA for 20 minutes, then transferred to Tube 2. Tube 2 was centrifuged 14,000 RCF for 10 minutes and then the supernatant collected for sequencing and identification, as described above.

Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS)

Using protocols previously validated [21–23] mass spectrometry analyses were conducted in order to ascertain the identity of proteins present in the *N. kuhlii* venom extract. Reduced alkylated (RA) and trypsinised (Tryp) samples were prepared for each venom protein extract. 6µg of the extracted proteins was aliquoted and vacuum-dried. The samples were reconstituted with 50 µL of 10% ACN, incubated at 37°C for 2 hours with the RA solution and lyophilised. The samples were then reconstituted in 50 µL of 40 mM ABC and separated into two 25 µL aliquots (RA and Tryp). RA

samples were dried overnight while Tryp samples were incubated with 500 ng trypsin at 37°C overnight and lyophilised. The samples were reconstituted with 20 μL 2.5% ACN and 1% FA and sequenced and identified, as described above. Full mass spectrometry sequence match data is presented in Supplementary File 2.

Phylogenetics

Proteomic hits identified as cystatins in the transcriptome database were blasted against GenBank to identify putative gene homologs. The resulting sequences were supplemented with (i) previously identified venom and non-venom cystatins sourced from GenBank and (ii) sequence data from previous studies that have described cystatin gene family evolution [24–26]. The resulting amino acid sequence data was aligned using the MUSCLE algorithm [27] implemented in MEGA6 [28] and a model of sequence evolution was selected by ModelGenerator [29]. The resulting model (WAG+I+Γ) was implemented in phylogenetic analyses using MrBayes v3.2 [30] on the CIPRES Science Gateway (http://www.phylo.org/sub_sections/portal). The cystatin gene tree was generated in duplicate using four chains for $1x10^7$ generations, sampling every 500^{th} cycle from the chain and using default settings in regards to priors. Tracer v1.4 [31] was used to estimate effective sample sizes for all parameters and to verify the point of convergence (burnin), with trees generated prior to this point discarded. This process was repeated for the hyaluronidase gene family following the identification of a hyaluronidase-like gene in the venom gland transcriptome of *N. kuhlii* and previous evidence of hyaluronidases (and their enzymatic activity) being identified in fish venoms [15,32–34].

Bioactivity testing

Ethical approval for experiments involving animals was obtained from the University of Queensland Animal Ethics Committee. All experiments were conducted in accordance the Animal Care and Protection Regulation Qld (2012), the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, 8th edition (2013) and the *International Association for the Study of Pain Guidelines for the Use of Animals in Research*. Male C57BL/6 mice (23 – 25 g) received an intraplantar injection of ammonium sulphate precipitated venom (n=3) or venom additionally acetone-precipitated (n=3) in a volume of 20 µl under isoflurane (3%) anesthesia. Paw erythema, swelling and nocifensive behaviour were quantified by a blinded observer. Paw thickness was measured using electronic calipers 1 hour after injection, and the number of paw lifts, licks and flinches were counted in 5 min intervals immediately after injection for 1 hour.

RESULTS AND DISCUSSION

Despite the abundance of venomous species, the venoms of fishes have received scant research attention, particularly when compared with their squamate reptile counterparts [2,6,25,35]. In part, fish venoms are neglected due to the inherent nature of such samples, in that fish venoms are heat labile, mucus rich and difficult to extract. To facilitate future work on fish venom samples, we describe a novel methodology to facilitate the extraction and processing of such samples for proteomic investigation. Venom protein extractions were cleaned and mucus removed using a multi-step protocol consisting primarily of precipitation with ammonium sulfate and acetone. Samples of the extracted venom proteins were taken after each of these steps to monitor the purity of the sample. We compared the samples collected during the cleanup process (post-ammonium sulfate precipitation and post-acetone treatment) on

a 1D SDS-PAGE gel (Figure 2). It was not possible to compare these samples with the initial protein extracted sample due to the extent of mucus contamination initially present preventing protein migration through the gel. Importantly, while the banding on the ammonium sulfate precipitated sample was identical to the ammonium sulfate and acetone precipitated sample, the banding on the acetone precipitation sample was stronger than the sample that was only ammonium sulfate precipitated, indicating a more accurate nanodrop reading due to the removal of contaminants (Figure 2).

We confirmed the retention of venom toxins during the cleanup process by testing the bioactivity of samples *in vivo*. The intraplantar injection of ammonium sulphate precipitated venom (20 µg) elicited immediate erythema and significant paw swelling (3.33 ± 0.25 mm) compared to the contralateral hind paw (1.47 ± 0.07 mm). Venom additionally acetone-precipitated retained activity and elicited edema in the ipsilateral (2.97 ± 0.27 mm) compared to the contralateral (1.50 ± 0.03 mm) paw, although erythema was less pronounced. Mild nocifensive responses, including spontaneous flinching, licking or lifting of the hind paw, were observed for both crude (23.0 ± 6.1 flinches /5 min) and acetone-precipitated venom (23.7 ± 8.7 flinches /5 min). These results demonstrate that the acetone precipitated venom retains bioactivity, thus indicating that denaturing is not a significant issue during the cleanup protocol.

We subsequently analysed the venom composition of *N. kuhlii* using our cleaned venom protein extract in a variety of proteomic analyses, namely 1D and 2D SDS-PAGE gel electrophoresis (Figure 3) and shotgun sequencing - all coupled to LC-MS/MS. To underpin the annotation of venom proteins detected by proteomics, we generated a venom gland transcriptome for *N. kuhlii*. Next generation sequencing of

this transcriptome resulted in 2.95 million reads, representing 1.47 million read pairs, with a mean read length of ~160 bp. The transcriptome assembly resulted in 4,584 contigs with an N50 of 602 bp. GO-term annotations of the assembled transcriptome revealed a variety of putative functions for the protein-encoding genes detected (Figure 4). Level 2 molecular functions were dominated by genes associated with 'binding' and 'catalytic activity', with both categories representing 79% of all annotated contigs (Figure 4A). Level 3 molecular functions were inherently more diverse, although, notably, predicted protein functions such as 'protein binding' and 'hydrolase activity' were well-represented (19% and 7% respectively) (Figure 4B).

We identified several protein types in the venom barb tissue of *N. kuhlii* via the annotation of detected proteins against the translated transcriptome database and the UniProt database (Figure 3 and Table 1). These proteins included haemoglobin subunit alpha, cystatins, galectin, ganglioside GM2 activator, glutathione S-transferase mu, leukocyte elastase inhibitor, transaldolase, ATP synthase, peroxiredoxin 6, nucleoside diphosphate kinase and type III intermediate filament. The relative abundance of the genes encoding these proteins, as detected by the venom gland transcriptome, are displayed in Figure 4C. The protein composition of *N. kuhlii* venom extract is therefore relatively complex, with multiple proteins identified within single gel spots and bands in many cases (Table 1). Notably, the vast majority of these proteins were identified by both the 1D and 2D gel methodologies, with relatively few additional proteins detected by the shotgun approach (Table 1). Whilst many protein types are clearly associated with conserved functions related to structure, transport and metabolism, a number of other proteins likely represent putative venom toxins.

We detected the presence of a galectin in the venom extract of *N. kuhlii*. The gene encoding this protein was highly expressed in the venom gland transcriptome (52nd most abundant contig of 4,584 and 2nd most abundant of those detected proteomically) and represents 12% of the *N. kuhlii* venome (venom protein composition) based on this transcriptomic data (Figure 4C). Galectins are lectins, which are broadly characterized as carbohydrate binding proteins. Other lectin-scaffolds, specifically C-type lectins, have been identified as toxic components in the venoms of snakes, *Lonomia* caterpillars and the bony fish *Thalassophryne nattereri*, where they exhibit diverse functions including anticoagulant, procoagulant, platelet-modulating, myotoxic and hemagglutination activities [36–38]. Whilst galectins are structurally unrelated to C-type lectins, a number of these proteins are apoptotic and pro- or anti-inflammatory [39,40]. Galectins are one of only two cell death-inducing ligands and function by binding to specific saccharide ligands located on the cell surface of gylcoproteins or glycolipids to initiate cell death [39]. The previously characterized functional activities of galectins are therefore relevant to a potential toxic venom function, particularly when considering that cell death is heavily implicated as the primary cause of pain disproportionate to the wound that characterises human envenomings by fishes [9].

Peroxiredoxin-6 (PRDX6) was another protein type of interest identified in the venom extract of *N. kuhlii*. This protein was encoded by a highly expressed transcript in the venom gland (4th most abundant contig of those detected proteomically) and, based on this expression data, represents 10% of the *N. kuhlii* venome (Figure 4C). Peroxiredoxins are an important family of antioxidant enzymes that are ubiquitous in

all organisms and are implicated in the control of cytokine-induced peroxide levels relevant to mediating cellular signal transduction [41]. Interestingly, PRDX6 is a bifunctional protein with one of its main functions resulting in the hydrolysis of lipids through phospholipase A_2 (PLA_2) activity [42]. PLA_2 enzymes have several known scaffolds that have been independently recruited into the venoms of different animals, including cephalopods, jellyfish, scorpions and snakes, and the derived toxic functions of PLA_2s include antiplatelet, myotoxic and neurotoxic activities [1]. Thus, similar to the PLA_2 activity observed in other venoms, PRDX6 may contribute to the toxic activity of *N. kuhlii* venom and should therefore be further investigated to characterise its functional activity and role in stingray envenoming.

Cystatins are a large superfamily of proteins that are potent inhibitors of cysteine proteinases, including papain and the cathepsins [43]. Cystatins have previously been identified in the venom of snakes and the spider *Chilobrachys jingzhao* [44,45] and also in the saliva of haematophagus ticks [46,47]. In snakes, their toxic role is ill defined, although venom cystatins may inhibit the defensive enzymes of the envenomed animal, thereby facilitating the toxic activity of other venom components [6]. Alternatively, cystatins may interfere with clotting processes by inhibiting cathepsin binding to components such as annexin-2, which ultimately serve as cell surface receptors for tissue plasminogen activator, plasminogen and plasmin [48–50]. We identified two cystatins in our proteomic analyses of *N. kuhlii* barb venom extract which, when combined, account for 19% of the venome based on the transcriptomic expression data (Figure 4C). Both cystatins were found to exhibit similarities to cystatin B-like proteins isolated from other animals. Despite their similar annotation, our phylogenetic analysis of the cystatin gene family revealed that the two cystatins

identified in *N. kuhlii* venom are non-monophlyetic, and they are also non-homologous to the cystatins previously recovered from other venomous animals (Figure 5). These results suggest that cystatin genes have been recruited in parallel to the venom gland of *N. kuhlii* and on at least four occasions in all venomous animals. Additional recruitment events seemingly account for the presence of cystatins in the saliva of haematophagus ticks (Figure 5).

Notably, we did not detect any previously characterised fish venom proteins (e.g. natterin, nattectin, stonustoxin – [38,51,52]) in our proteomic or transcriptomic analyses. These results are perhaps unsurprising considering (i) the phylogenetic distance between *N. kuhlii* and the bony fishes from which these toxins have previously been identified and (ii) the likely independent evolution of the venom systems of cartilaginous and bony fishes. Perhaps more surprising was the absence of hyaluronidase and the small toxic peptides orpotrin and porflan in our *N. kuhlii* venom extract. These proteins/peptides were previously molecularly characterised (or in the case of hyaluronidase, its activity detected) in the venom of freshwater *Potamotrygon* stingrays [15–17]. Whilst we did not detect genes encoding orpotrin- or porflan-like peptides in the *N. kuhlii* venom gland transcriptome (or venom proteome), we did identify a hyaluronidase-like gene expressed in the venom gland of *N. kuhlii*; although the corresponding protein was absent from the venom proteome. However, phylogenetic analysis of the *N. kuhlii* hyaluronidase-like gene revealed that it was non-monophyletic to hyaluronidases previously molecularly characterised from fish venoms [32–34] (Figure 6). These results suggest that the detected *N. kuhlii* hyaluronidase-like gene is not a venom component, and therefore explain its absence from the venom proteome. Further validating these results is the previously described

absence of hyaluronidase activity in venom extracts isolated from the marine stingray *Dasyatis guttata* [53], a species which, until recently, was viewed as congeneric with *N. kuhlii* [54]. This data suggests that inter-specific variation in toxin composition, as observed in other venomous taxa [55–57] also exists between different stingrays. Such variation may be the result of (i) the unique recruitment of some toxin types into the venom of some species, (ii) the loss of toxin types from the venom of some species, or (iii) potentially as the result of the independent evolution of venom systems in different stingray lineages.

Our study represents the first broad characterisation of the venom composition of a cartilaginous fish and the first combined proteomic and transcriptomic study of the venom of any fish. We describe and utilise a novel protein extraction methodology that overcomes many of the undesirable aspects of studying fish venom and will hopefully enable research on such animals to join contemporary scientific circles. The identification of a variety of proteins that are novel to venom highlights the untapped potential of fish venoms and their toxins, and therefore such samples may be valuable for future biodiscovery projects. We next intend to apply the approach utilised here to different fish lineages to investigate the evolutionary history and composition of venom found in these understudied venomous vertebrates.

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SUPPLEMENTARY MATERIAL

Supplementary file 1 nucleotides

Supplementary file 2 mass spectrometry

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Table 1. Protein types identified in the barb venom gland extract of *N. kuhlii*

Protein Type	1D bands	2D spots	Shotgun	UniProt match	Known functions
60S acidic ribosomal protein			✔	K4GJD9	Elongation in protein synthesis (UniProt)
ATP synthase	5	1, 2, 3, 4, 6	✔	Q9PTY0	Generating ATP [58]
Coronin			✔	F1QDY7	Actin binding protein [59]
Cystatin	9	6	✔	Q28988, J7FQE8	Cysteine proteinase inhibition [43]
Cytochrome C	9		✔	Q6DKE1	Electron carrier activity (UniProt)
Ferritin			✔	Q801J6	Important in iron homeostasis (UniProt)
Galectin	1, 4, 5, 6, 7, 8, 9	3, 4, 6, 7	✔	H2UTD9	Apoptotic, pro-/anti-inflammatory [39,40]
Ganglioside GM2 activator	8	2, 3	✔	K4FYQ1	Unknown activity
Glutathione S-transferase mu		4, 5		Q9TSM5	Cellular detoxification [60]
Hemoglobin subunit alpha	2, 4, 5, 6, 7, 8, 9	1, 2, 3, 4, 5, 7	✔	P56691	Antimicrobial [61]
Leukocyte elastase inhibitor	2, 3, 9	1	✔	R0LF52	Inflammation [62]
Nucleoside diphosphate kinase		7	✔	G3HBD3	Regulatory functions [63]
Peroxiredoxin 6	1	2, 3, 4		K4FY71	Antioxidant functions [64]

Transaldolase	3	1	✔	Q28H29	Glucose metabolism [65]
Type III intermediate filament	9	1	✔	P23729	Structural [66]
Voltage-dependent anion channel	4		✔	Q9IA66	Diffusion of small hydrophilic molecules (UniProt)

FIGURE LEGENDS

Figure 1. The blue-spotted stingray *Neotrygon kuhlii* and its venom delivery system. A) A photograph of a collected specimen of *N. kuhlii* from Moreton Bay, Australia. The arrow highlights the location of the venom delivery system (barb) on the caudal appendage. B) Stereo microscopy photographs of the retroserrate venom spine of *N. kuhlii* at different magnifications. Left – ventral view showing the median ventral ridge to which the venom glands (not present here) flank; Right – dorsal view.

Figure 2. 1D SDS-PAGE gel profile of *N. kuhlii* barb venom protein extract at different stages of the protein cleanup protocol. AMS = ammonium sulfate and AcOH = acetone. The gel was stained with Colloidal Coomassie Brilliant Blue G250.

Figure 3. 1D and 2D SDS-PAGE gel profiles of *N. kuhlii* barb venom protein extract. A) 1D SDS-PAGE gel profile highlighting the bands that were selected for in-gel digestion and protein identification. B) 2D SDS-PAGE gel profile highlighting the spots that were selected for in-gel digestion and protein identification. The numbers in each gel refer to the proteins displayed in Table 1. The gels were stained with Colloidal Coomassie Brilliant Blue G250.

Figure 4. GO-term classification of the assembled and annotated *N. kuhlii* venom gland transcriptome. A) Level 2 and B) level 3 GO-term analysis of the annotated contigs. C) The relative abundance of proteins present in the *N. kuhlii* venome (venom proteome) calculated by transcriptomic expression levels.

Figure 5. Phylogenetic relationship of the cystatin gene family demonstrating the parallel recruitment of these proteins for a role in venom. Branches highlighted in red indicate cystatins that have been recovered from the venom glands of venomous animals (snake, spider and stingray) or the saliva of haematophagus animals (tick). Note the apparent parallel recruitment of different cystatins into the venom of *N. kuhlii*. Black circles represent nodes with Bayesian posterior probabilities (Bpp) of 1.00 and grey circles Bpp of >0.95.

Figure 6. Phylogenetic relationship of the hyaluronidase gene family demonstrating that the *N. kuhlii* hyaluronidase-like venom gland transcript is paralogous to previously characterised hyalruonidase from fish venoms. Branches highlighted in red indicate hyaluronidases that have been recovered from the venom glands of venomous animals (snakes and bony fish). Black circles represent nodes with Bayesian posterior probabilities (Bpp) of 1.00 and grey circles Bpp of >0.95.

Conflict of interest

None

SIGNIFICANCE

- These results show an efficient manner for removing mucus from fish venoms
- These results are the first insights into the evolution of proteins present on stingray venom barbs

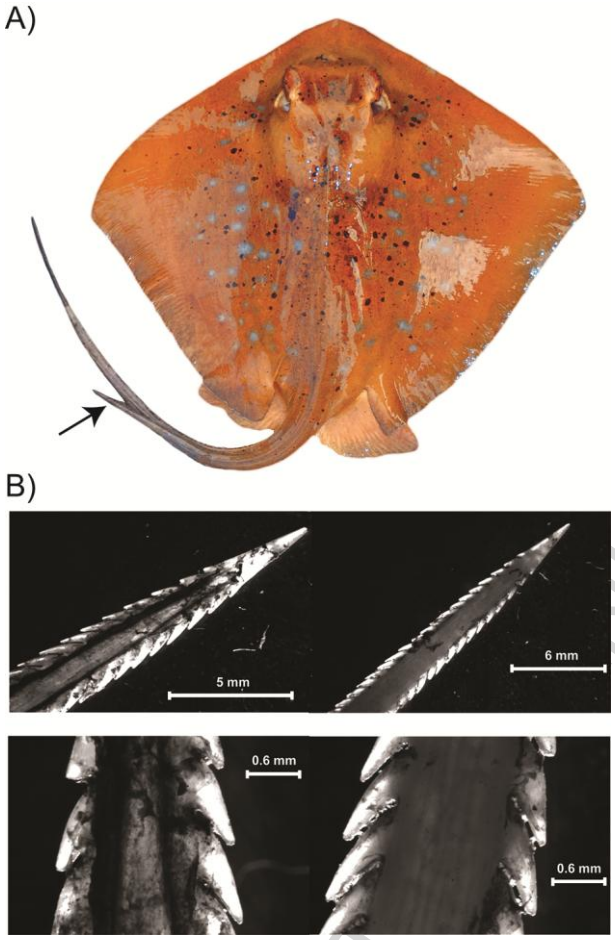

Figure 1

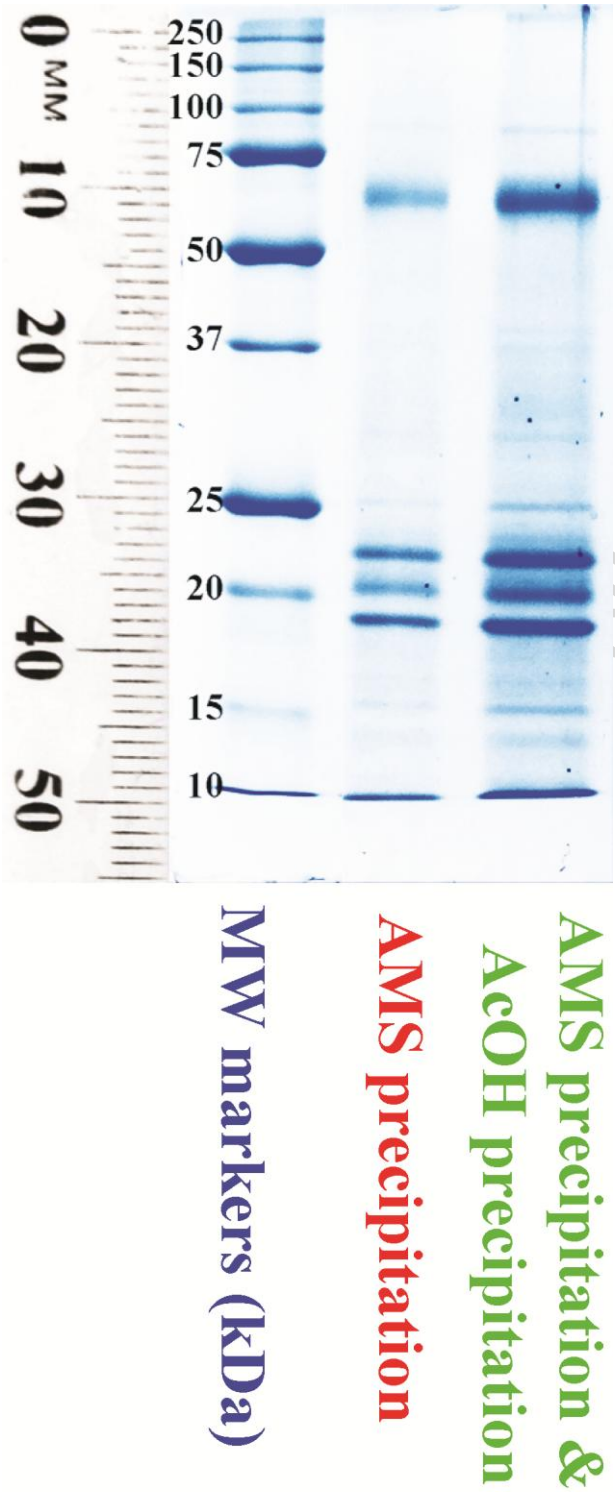

Figure 2

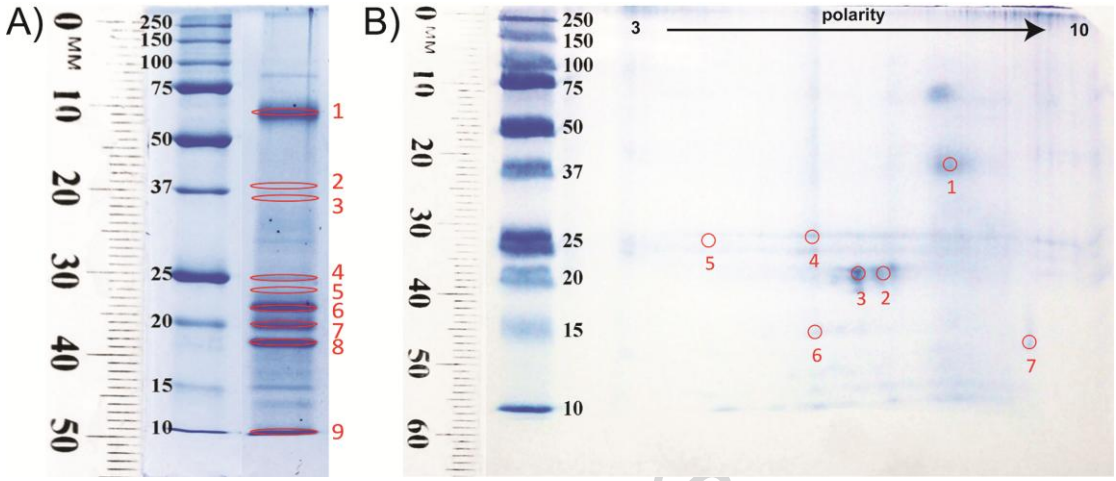

Figure 3

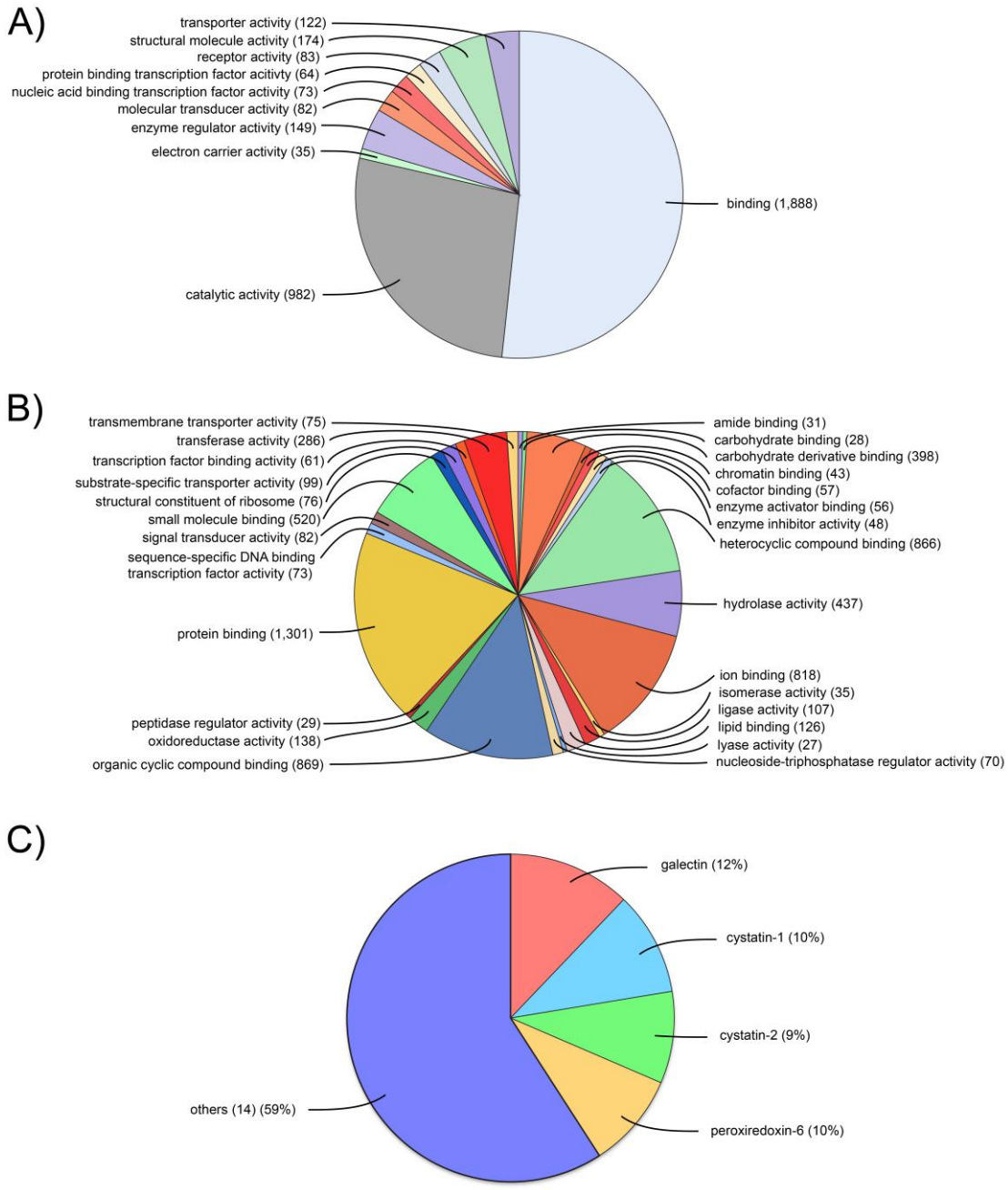

Figure 4

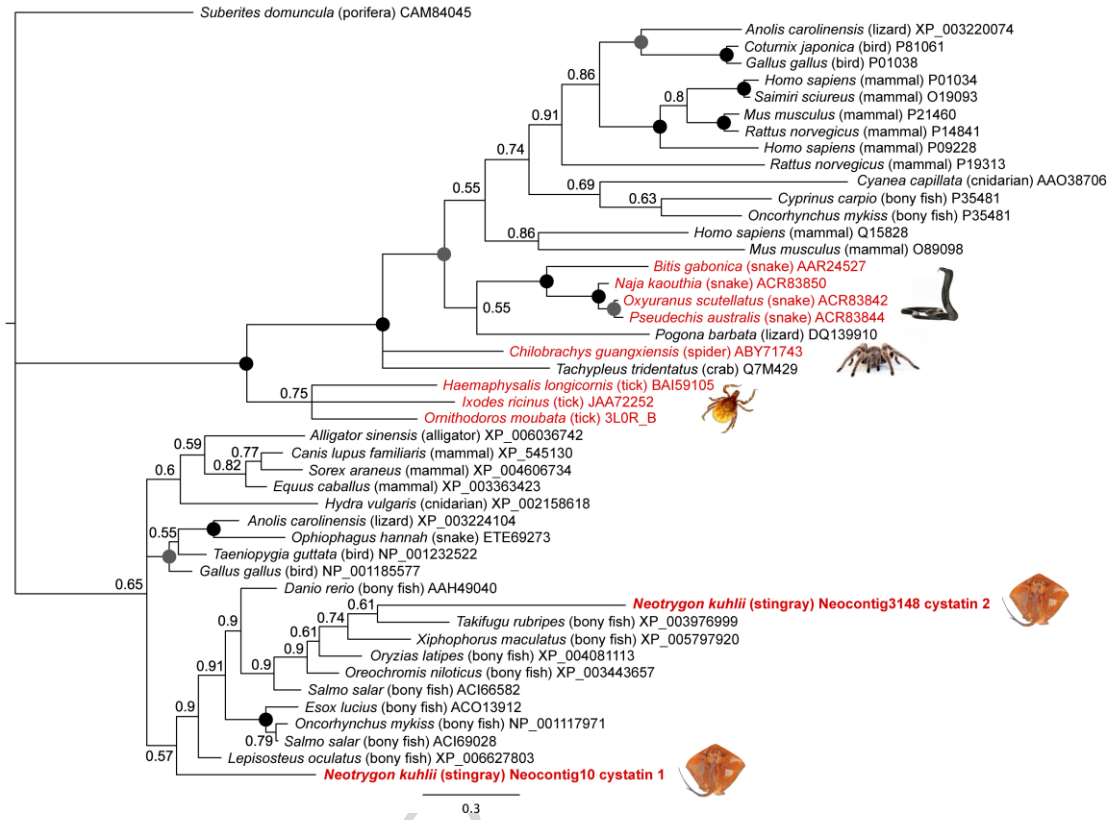

Figure 5

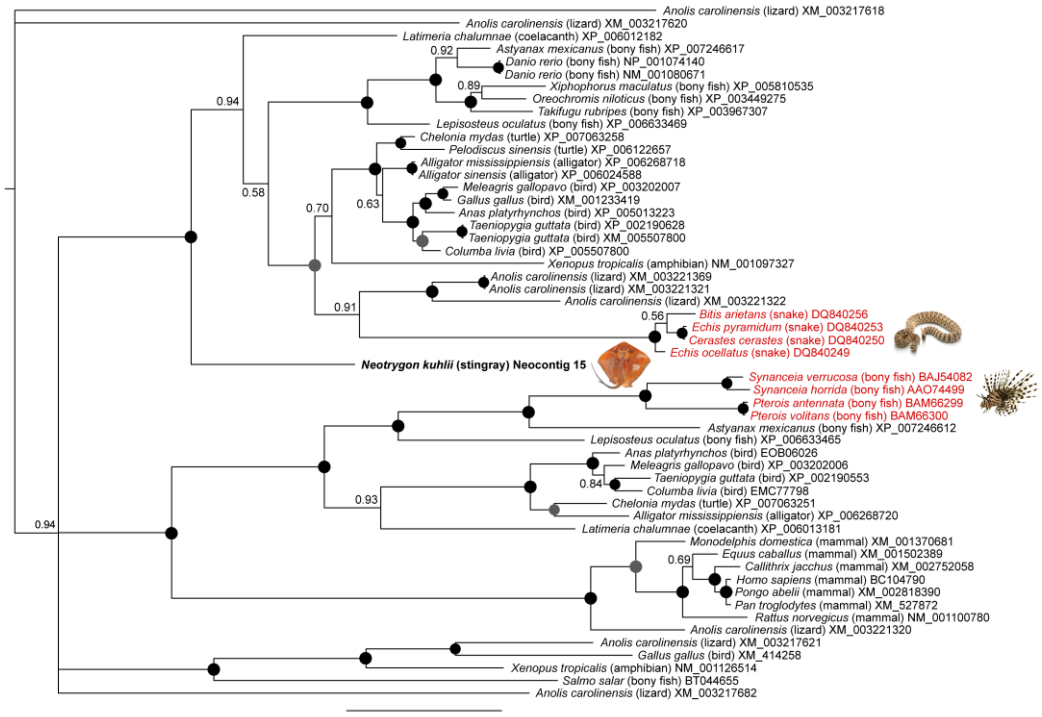

Figure 6

Graphical abstract

HIGHLIGHTS

- A combined chemical cleanup approach can remove mucus from stingray venom barb protein extract
- Stingray venom barb protein extract is complex proteomically
- The first sequencing of a stingray venom barb transcriptome