RESEARCH ARTICLE

Plectreurys tristis venome: A proteomic and transcriptomic analysis

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ABSTRACT

Spider venoms are complex cocktails rich in peptides, proteins and organic molecules that collectively act to immobilize prey. Venoms of the primitive hunting spider, *Plectreurys tristis,* have numerous neurotoxic peptides called "plectoxins" (PLTX), a unique acylpolyamine called bis(agmatine)oxalamide, and larger unidentified protein components. These spiders also have unconventional multi-lobed venom glands. Inspired by these unusual characteristics and their phylogenetic position as Haplogynes, we have partially characterized the venome of *P. tristis* using combined transcriptomic and proteomic methods. With these analyses we found known venom neurotoxins U_1-PLTX-Pt1a, U_3-PLTX-Pt1a, and we discovered new groups of potential neurotoxins, expanding the U_1- and ω-PLTX families and adding U_4-through U_9-PLTX as six new groups. The venom also contains proteins that are homologs of astacin metalloproteases that, combined with venom peptides, make up 94% of components detected in crude venom, while the remaining 6% is a single undescribed protein with unknown function. Other proteins detected in the transcriptome were found to be members of conserved gene families and make up 20% of the transcripts. These include cDNA sequences that match venom proteins from *Mesobuthus* and *Hottentotta* scorpions, *Loxosceles* and *Dysdera* spiders, and also salivary and secreted peptide sequences from *Ixodes*, *Amblyomma* and *Rhipicephalus* ticks. Finally, we show that crude venom has neurotoxic effects and an effective paralytic dose on crickets of 3.3μg/gm.

KEYWORDS: Spider, venom, astacin metalloprotease, neurotoxin, PD_{50}, cDNA library, Orbitrap mass spectrometry

INTRODUCTION

Spider venoms contain thousands of bioactive components that are used primarily for immobilization during prey capture. The majority of the components are peptide neurotoxins or toxic proteins. Methods of prey entrapment and immobilization vary widely among the 44,000 described species of spiders. Comparative analyses are revealing that venom compositions also vary widely across spiders. Neurotoxic venom peptides are of particular interest for potential applications as insecticides or pharmaceuticals because of their targeted specificity on neuronal membrane proteins (Escoubas et al, 2000; King, 2011; Windley et al, 2012; King and Hardy, 2013). These peptides can cause excitatory and/or inhibitory effects on target animals with zoological variability in potency (mammals, insects, *etc*.). While many groups are actively working on spider venom characterization, much of the vast phylogenetic space of spiders remains largely unexplored. Expanding our understanding of venom composition leads to discovery of novel toxins and helps provide details that allow inference of the scale of variability among spider venoms.

In this work we focus on *Plectreurys tristis*, a species from which venom peptide neurotoxins have previously been discovered and characterized (Branton et al, 1987; Branton et al, 1993;

Quistad et al, 1993; Quistad and Skinner, 1994; Leisy et al, 1996; Zhou et al, 2013). These spiders are of interest because they are a distinct and old family with a fossil representative that is 165Ma (Selden and Huang, 2010). Moreover, individuals have large, unusual multi-lobed venom glands (Millot, 1935), in contrast to typical tubular, sausage shaped glands found in other spiders. The small family Plectreuridae contains 31 species that make up only two extant genera and are found in desert regions of North America, Mexico and Cuba (Platnick, 2014). They live in haphazard webs built under rocks, bark and debris (Carpenter et al, 1990; Selden and Huang, 2010) and their feeding biology has not been studied as far as we know. Plectreurid venom does not seem to be harmful to humans; there exists only one case report of human envenomation, which caused pain, edema, slight pallor and numbness, and complete recovery occurred within a few hours (Carpenter et al, 1990). In contrast, this venom is quite toxic in lepidopteran insect larvae with individually purified peptides showing a broad range in potency (Quistad and Skinner, 1994). These peptides are inhibitory on Ca^{2+} channels and/or excitatory on Na^+ channels, affecting synaptic transmission patterns in *Drosophila* (Branton et al, 1987; Zhou et al, 2013). Although several peptides have been analyzed and shown to have these activities, only two have been extensively studied: ω-PLTX-Pt1a (Branton et al, 1987) and δ/ω-PLTX-Pt1a (Zhou et al, 2013). Bis(agmatine)oxalamide (Plt-I) was identified in *P. tristis* crude venom using HPLC, and is the only component that exists in a molecular weight region that contains many forms of acylpolyamines in other spiders; it is also devoid of insecticidal and fungicidal activity (Quistad et al, 1993). To date, only small venom components have been analyzed in plectreurid venom, even though larger protein products have been detected as peaks in HPLC venom separations (20–70kDa, Branton et al, 1987; Zhou et al, 2013).

The goal of this study was to expand our understanding of the composition of peptides and proteins that make up the venome (transcriptome and proteome constituents) of *P. tristis* spider venom. To our knowledge, this is the first broad-scale molecular study of plectreurid spider venom. We have identified a diverse set of astacins, several new groups of venom toxins and many other polypeptides. In addition, we report neurotoxic effects and potency of *P. tristis* crude venom (PD_{50} values) on crickets, a likely source of natural prey. As a distinct Haplogyne family, these data provide an important phylogenetic landmark for comparative analyses of spider venom diversity.

MATERIALS AND METHODS

We used venom and venom gland tissue isolated from the same five female *P. tristis* spiders. All spiders were collected in the field by Charles Kristensen (Spider Pharm Inc) in May, 2012 (Yarnell, AZ) under or surrounding tree bark from a single, small locality. We used electrical stimulation to extract venom as in Binford and Wells (2003). Additional venom was purchased from Spider Pharm for use in bioassays on crickets.

Transcriptome analysis
cDNA library construction
To up-regulate venom transcripts, venom was extracted from the five spiders described above across two consecutive days (three milked on one day and the other two the following day) with a goal of increasing the breadth of transcripts we captured. On the same day (three and four days after extraction), all five spiders were anesthetized using CO_2 and venom glands were extracted and immediately flash-frozen in liquid nitrogen. While transcriptional timing is not known in plectreurid spiders, this window of time has been successful for isolating venom gland mRNAs from other Haplogyne spiders (Binford et al, 2005; Zobel-Thropp et al, 2014). Total RNA was isolated using the ChargeSwitch Total RNA Cell kit (Invitrogen).

We constructed the venom gland cDNA library using the SMART cDNA library construction kit (Clontech). Details of all procedures including library construction, cDNA packaging, screening and sequence analysis are the same as in Zobel-Thropp et al (2014). For this library, we used 0.2μg of total RNA for first strand synthesis of cDNA and followed the manufacturer's protocol for library construction. The library titer was $1.2x10^6$pfu/ml. We screened 1,717 clones using PCR and sequenced 451 cDNAs ≥500bp on an Applied Biosystems 3730 Analyzer at the University of Arizona (USA). We trimmed and assembled all sequences using Sequencher 5.1 (Gene Codes Corp); due to chromatogram ambiguity, we discarded 144 sequences, resulting in 307 high quality sequences for transcriptomic analysis.

Bioinformatics
Our analysis pipeline is illustrated in Figure 1. All database searches and sequence analyses including domain and motif searching, protein features, and alignments were conducted as in Zobel-Thropp et al (2014). All nucleotide sequences were submitted to tBLASTx searches in the ArachnoServer spider toxin database (Herzig et al, 2011; http://www.arachnoserver.org/blastForm.html), the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) and also searched against our database containing sequences from several Haplogyne venom gland cDNA libraries constructed in the Binford lab (unpublished). Hits were considered significant if e-values were ≥10^{-5}. Eighty-five sequences were omitted because we interpreted them to be contaminants; they hit CU223764, *Populus* cDNA from leaves in the NCBI database and translated into repetitive runs of amino acid segments without an open reading frame. We assembled the remaining nucleotide sequences (222 total) into sequence clusters using 99% and 85% threshold identities with a minimum 20bp overlap. The latter is used for predicting functions of sets of homologs (Figure 1B), as sequences within these clusters individually fell into the same functional category. To quantitatively assess how thoroughly we sampled the full diversity of transcripts in this venome, we applied a biodiversity analysis designed for assessing sampling of species diversity, following Remigio and Duda (2008) (www.alyoung.com/labs/biodiversity_calculator.html).

We looked more closely at the sequences that did not hit matches in searches to identify other sequence characteristics that might give clues to their general function. Protein coding sequences were considered full-length "open reading frames" (ORFs) if they contained a methionine, a stop codon and consisted of ≥30aa in at least one translated
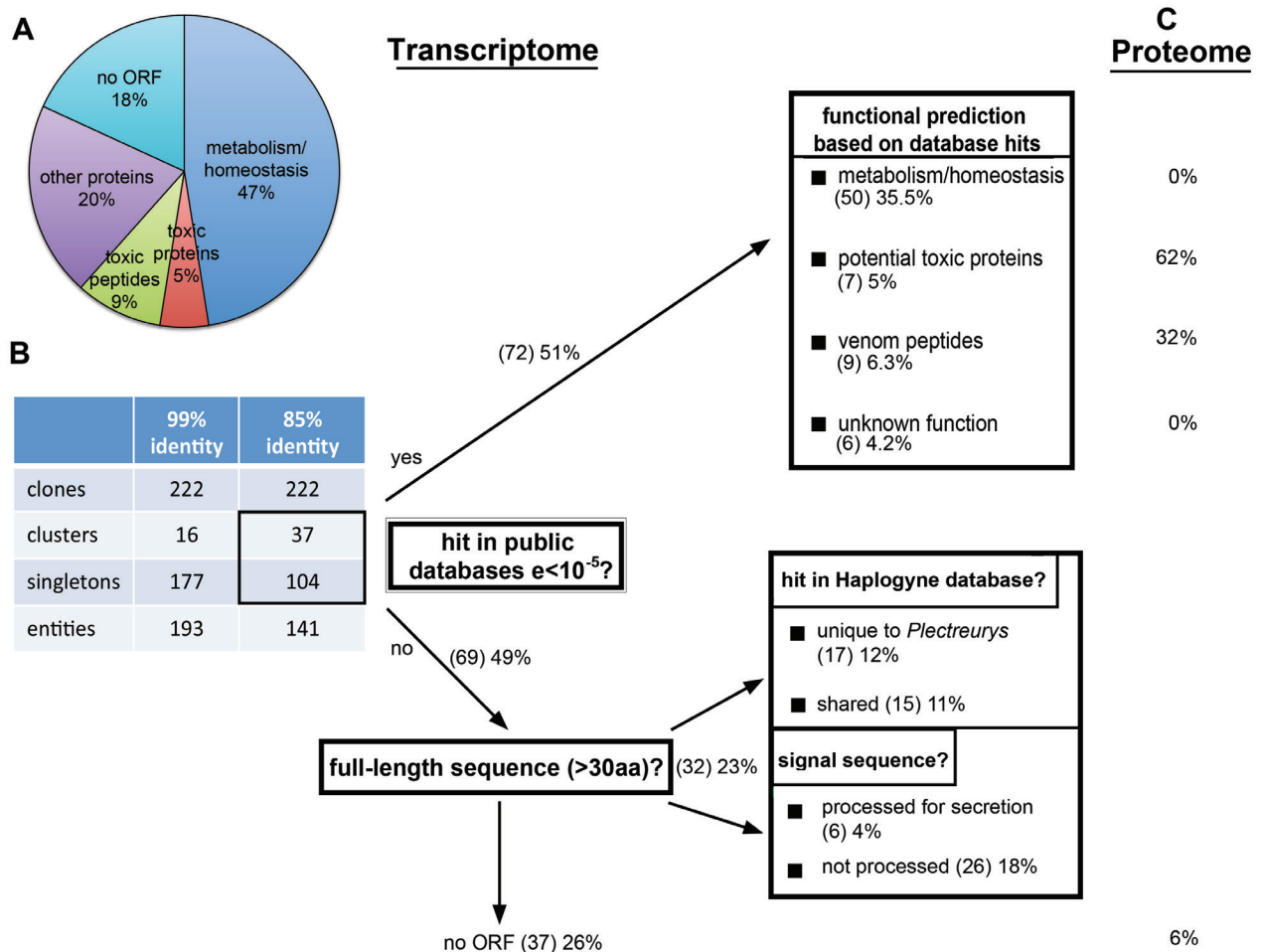
A

Transcriptome

C
Proteome

Pie chart (A):
- no ORF 18%
- metabolism/homeostasis 47%
- other proteins 20%
- toxic peptides 9%
- toxic proteins 5%

functional prediction based on database hits
- ■ metabolism/homeostasis (50) 35.5% — 0%
- ■ potential toxic proteins (7) 5% — 62%
- ■ venom peptides (9) 6.3% — 32%
- ■ unknown function (6) 4.2% — 0%

B

	99% identity	85% identity
clones	222	222
clusters	16	37
singletons	177	104
entities	193	141

(72) 51%

yes

hit in public databases e<10^{-5}?

no (69) 49%

full-length sequence (>30aa)? (32) 23%

hit in Haplogyne database?
- ■ unique to *Plectreurys* (17) 12%
- ■ shared (15) 11%

signal sequence?
- ■ processed for secretion (6) 4%
- ■ not processed (26) 18%

no ORF (37) 26%

6%

Figure 1. Overview of *Plectreurys* venome analysis. **A.** cDNA sequences are divided based on overall transcript abundance into five general categories based on database hits (e≤10^{-5}) before clustering and categorizing functional prediction. **B.** Table: the 99% and 85% identity threshold assemblies respectively identify redundant and homologous sequences within the library. Flowchart: clusters and singleton sequences from the 85% threshold analysis were initially divided based on significant tBLASTx hits (e≤10^{-5}) in Arach-noServer or NCBI nt/nr databases. General function prediction was based on sequences hit in the databases. Sequences that did not hit anything in public database searches were searched against a Haplogyne database (Binford et al, unpublished) to identify components that were unique to *Plectreurys,* and those that shared homology among Haplogyne spiders. We looked for signal peptide cleavage sites within all ORF sequences using SignalP (http://www.cbs.dtu.dk/services/SignalP/). **C** The percentage of proteins detected by mass spectrometry from the crude venom was calculated based on the number of tryptic peptides that matched translated sequences of coding proteins divided by the total number of peptides detected in the digest.

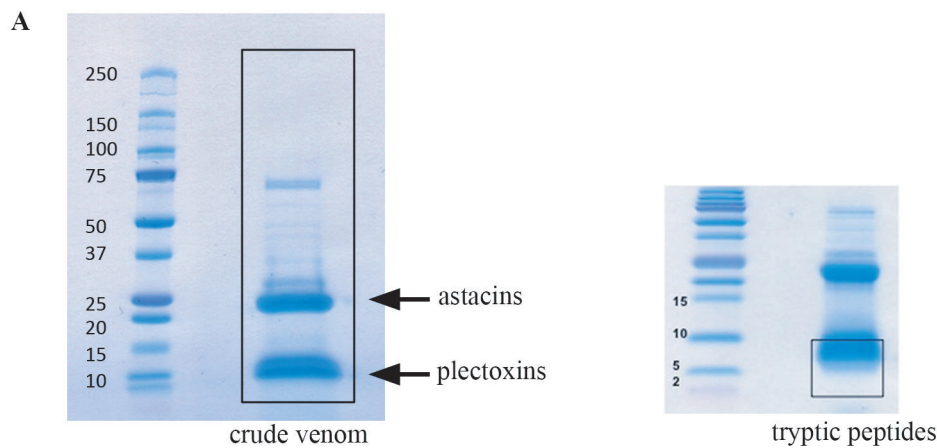frame. Three clusters and 34 singleton sequences were deemed "no ORF" (Figure 1B).

All nonredundant sequences >200bp were submitted to GenBank (KJ124597-KJ124723, KJ561453, and KJ561454).

Proteome analysis
LC-MS/MS detection of tryptic peptides from crude venom
Venom from the same individuals used for the transcriptome was dissolved in a standard buffer (5mM CaCl$_2$/50mM Tris, pH 8.0), and total protein was quantified using Bradford Coomassie protein reagent (Pierce). We submitted 100µg to the Arizona Proteomics Consortium (University of Arizona) for proteomic analysis. The venom-expressed proteins were analyzed from an in-gel tryptic digest (Figure 2) with LC-MS/MS using an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) as in Zobel-Thropp et al (2014). In brief, samples were injected onto a Proxeon Easy nano-HPLC (Proxeon, Odense) precolumn (100µm, 2cm,

Fisher Scientific) and separated on a C18 column (75µm, 10cm, Fischer Scientific) at a flow rate of 300nl/min over a 145-minute gradient of 5–40% solvent B (A = water, B = acetonitrile, both with 0.1% (v/v) formic acid). Eluate was delivered by a nanospray source (Nanomate, Advion) with a voltage of 1.85kV to the Orbitrap Velos. Survey scans were acquired at 60,000 resolution and the top 14 *m/z* values were selected for CID (width = 2amu) at 35% relative energy in the Velos linear trap and then placed on an exclusion list for 45sec. Resulting MS/MS data was searched using Sequest on Discoverer (v. 1.3.0.339, Thermo Fisher Scientific) against masses of theoretical fragments from a database that included our plectreurid transcriptome sequences, unpublished sequences from multiple Haplogyne venom gland tissue cDNA libraries translated in the forward three frames, and all chelicerate sequences in NCBI (downloaded 11/17/13), totaling 93,243 sequences. Matches required <10ppm precursor error. The reversed database was searched to provide quality scores for FDR filtering. The

Figure 2. Proteomic analysis of *Plectreurys* venom. **A**. Protein and peptide gels were used to visualize the venom proteome and to enrich for smaller polypeptides, respectively. Left panel, 4-20% Tris-glycine SDS-PAGE of crude venom (15 μg). Arrows point to regions corresponding to predicted astacin and venom peptide sizes based on transcriptome data. Right panel, 16.5% Tris-tricine SDS-PAGE of crude venom (25μg). Boxed areas indicate excised regions that were digested with trypsin (Princeton Separations) in the presence of ProteaseMAX (Promega) following the manufacturer's protocol. All extracted peptides were desalted using OMIX C18 tips (Agilent Technologies), combined, and analyzed by LC MS/MS. **B**. Tryptic peptides that were positive hits against our cDNA database with ≥96% identity are listed and categorized into three groups based on general function. For each peptide, the percent coverage was calculated by dividing the number of amino acids in the peptide detected by the number of amino acids in the open reading frame of the corresponding cDNA sequence (Met through *stop); the superscript "nfl" indicates that the corresponding sequence is not full length. The cDNA sequence for clone 1531 is 77% identical to cDNA 1370, but corresponds only to the C-terminal 100 amino acids of the sequence, so we are not able to calculate size or percent coverage. Alignments of astacin and plectoxin sequences are presented in Figures 3 and 4, respectively.

Sequest output was organized in Scaffold (Searle, 2010). Proteins were identified with high confidence of 1% false discovery rate (FDR) and 3 unique peptides; U_6-PLTX-Pt1a (Table 1) was identified with a single peptide and 5% FDR.

Bioassays

To assess potency of *Plectreurys* venom, we quantified the dose at which 50% of crickets (*Acheta domestica*) injected were paralyzed after 60min (PD_{50}). We diluted venom in physiologically buffered lepidopteran saline solution (5mM KH_2PO_4/100mM KCl/4mM NaCl/15mM $MgCl_2$/2mM $CaCl_2$, pH 6.5) and established a dosage range distributed around the PD_{50} by performing preliminary injections. Experimental doses were 0.2, 0.4, 0.8, 1.2 and 1.6μg of crude venom protein with three replicate assays each, on 20 crickets per dose, by injection into the dorsal mesothorax using a PB-600 dispenser with a gastight syringe (Hamilton Co). With each replicate, we injected an additional 20 control crickets with 0.4μl of saline, a volume equal to the largest dose volume. Paralysis was scored every 10min for 1hr based on the ability of a cricket to right itself. PD_{50} values were generated for both absolute amounts of venom injected

Table 1. *Plectreurys* venom peptide analysis. Small cysteine-rich venom peptide groups are listed in order of ascending predicted full-length (FL) sequences from amino acid sequence alignments. The Ux-PLTX nomenclature was assigned based on nonredundant full-length (FL) sequences from amino acid sequence alignments. We used compute pI/MW from ExPASy (http://web.expasy.org/compute_pi/) to predict size (MW) and overall charge (pI) of mature protein and peptide sequences. All peptide lengths were determined using SpiderP (Wong et al, 2013); mature sequences were determined to the best of our abilities using SpiderP and proteomic results. Cysteine skeleton patterns are listed to show characteristic features of known venom peptide neurotoxins: a dashed line (–) indicates the presence of more than one amino acid surrounding the cysteine residue, primary structural motifs –C6C-CC-, -C-C-CC- (PSM), and –C1C- (ESM) are indicated if present. A checkmark indicates that one or more peptides from these groups were detected in the proteome, which are **bold** in the predicted mature sequence. The underlined sequences are C-terminal extensions following the terminal cysteine residue.

Top hit in each category of ArachnoServer, NCBI (EST), and Haplogyne (cDNA) database if e<10^{-5}	Proposed *Plectreurys* toxin name (PLTX)	# of cDNA seqs	# of unique full-length (FL) seqs	Size of FL/pro/mature peptide (#aa)	Average MW of peptides in the group (Da)	Detected using LC MS/MS	Predicted sequence of mature peptide	Predicted to be a knottin?	Cysteine skeleton pattern
Sicarius cDNA	U$_4$-PLTX-Pt1a	1	1	81/62/36	3992.65		LSCRSVGSCQRRSDCCKTCSYKKGCTNPSHCKKYCA	yes	-C-C-CC-C-C-C-
ω-plectoxin-Pt1a, *Physocyclus* cDNA	ω-PLTX-Pt1b	1	1	84/64/47	5228.87	✓	ADCSATGDTCDHTKKCCDDCYTCRCGTPWGANCRCDYHKMRCDTGKK	yes	-C6C-CC-C-C1C-C1C-C-
U$_1^1$-plectoxin-Pt1a, *Physocyclus* cDNA	U$_1$-PLTX-Pt1g	1	1	79/59/46	5305.06	✓	AGKCFGWQEDCDGRSPCCDDCVICQCNVIGQNCRCNRTRPSCLSGR	yes	-C6C-CC-C-C1C-C1C-C-
U$_3$-aranetoxin-Ce1a, *Physocyclus* cDNA	U$_5$-PLTX-Pt1a	2	1	89/70/70	7584.58	✓	QKYCDCTADQCCVRATGYSSGRCASLAGESEFCGFLEADGTHYRTYCP-CKPGLQCKLMYAGTSPYGCVKE	no	-C-C-CC-C-C-C1C-C-C-
ω-plectoxin-Pt1a, *Physocyclus* cDNA	U$_6$-PLTX-Pt1a	1	1	92/70/70	8145.36	✓	KQCGVDYQTCDVNEPPCCECYECSCPTQSQDSNCQCVYLKDKCGTG-KIRCNMKRFWDFNFRMLNKCECCV	yes	-C6C-CC-C-C1C-C1C-C-C-C-CC-
U$_{33}$-theraphotoxin-Cj1c, *Physocyclus* cDNA	U$_7$-PLTX-Pt1a	1	1	102/82/78	8323.44	✓	ECTKASDCGENECCARSHKSQQAYCRPLKTLGTHCDTKVKADGNGVY-SCHCPCVKGLECFTRTGSRGHCRLAGAVTPA	yes	-C-C-CC-C-C1C-C-C-
	U$_7$-PLTX-Pt1b	1	1	102/82/78		✓	ECTKASDCGENECCARSHKSQQAYCRPLKTLGTHCDTKVKADGNGVY-SCHCPCVKGLECFTRTGSRGHCRLAGTVTPA	yes	-C-C-CC-C-C1C-C-C-
	U$_8$-PLTX-Pt1a	2	1	117/98/98	10265.70		QSCGWRTCSTDQCCVRIPWRSRCLPRGGLGEMCGSPPYNCPCQDGY-DCVPMLFNLGTCRIRSTTASPNVTTIQTAVPTGMTSTQATPEIASPTTMTPT	no	-C-C-CC-C-C1C-C-C-
U$_{33}$-theraphotoxin-Cj1c, *Loxosceles* cDNA	U$_8$-PLTX-Pt1b	2	1	117/98/98			QSCGWRTCSTDQCCVRIPWRSRCLPRGGLGEICGSPPYNCPCQDGYDCVPM-LFNLGTCRIRSTTASPNVTTIQTAVPTGMTSTQATPETASPTTMTPT	no	-C-C-CC-C-C1C-C-C-
	U$_9$-PLTX-Pt1a	3	1	121/106/106			ECSTNAECSKNIWCCVEIGGFGGFGSSRKTCRNMGERGNCNPRYQTGDGP-FRGFCPCNNALKCERESIEVPGPFRPPISRVFRCPDSSTYSTTTTSNPFSFGFGK	no	-C-C-CC-C-C1C-C-C-
U$_3$-aranetoxin-Ce1a, *Physocyclus* cDNA	U$_9$-PLTX-Pt1b	1	1	146/127/106	11643.04		ECSTNADCKKDIECCVEIGGFGGFGSSRKTCRNMRERGNMCNPRYQTGDGP-FRGFCPCNNTLKCERESIEVPGPFRPPISRIFRCQDSSTSSTTTTSNPFSFGFGK	no	-C-C-CC-C-C1C-C-C-
	U$_9$-PLTX-Pt1c	3	1	146/127/106			ECSTNADCKKDIECCVEIGGFGGFGSSRKTCRNMGERGNMCNPRYQTGDGP-FRGFCPCNNTLKCERESIEVPGPFRPPISRIFRCQDSSTSSTTTTSNPFSFGFGK	no	-C-C-CC-C-C1C-C-C-
		19	12						

(μg) and for weight standardized dose (μg/gm cricket) using the EPA Probit Analysis Program for calculation of LC/EC values (version 1.5 SAS).

RESULTS AND DISCUSSION

We analyzed 222 sequences from a *Plectreurys tristis* venom gland tissue cDNA library (Figure 1). Within this dataset, 193 sequences are unique (at the 99% identity threshold), constituting 87% of the transcriptome. Clustering nucleotide sequences with an 85% identity threshold resulted in 37 clusters all of which contained sequences that were easily aligned, suggesting that clusters group sets of closely related paralogs or allelic variants. For functional prediction classification, we assume general function is based on significant matches with database sequences (e≤10^{-5}) and that function is consistent within clusters; however, all sequences were searched against all databases. At this level, we identified a total of 141 "entities", which we define as the combined total of clusters and singletons (Figure 1B). The Simpson index from a diversity curve analysis on the data from the 99% assembly was extremely small (0.0019), and a rarefaction curve did not plateau. There is also a high percentage of singleton sequences (177/222 = 80%). All of these patterns are consistent with the complete set of possible transcripts not being exhaustively searched (Remigio and Duda, 2008). While we discovered many new *Plectreurys* venom components, we consider this a partial trancriptome and acknowledge that more thorough analysis should yield more diversity of transcripts.

Sixty-four percent of all 222 sequences had significant matches in GenBank or ArachnoServer (e≤10^{-5}). Hits included proteins that are likely involved in nonvenomous "housekeeping" functions (metabolism/homeostasis), toxic proteins, venom peptides and other polypeptides for which we were not able to predict general function (Figure 1A). Of the remaining 69 entities that did not hit sequences in any databases, 32 were full length with an initiating methionine and a stop codon. For these we searched for sequence motifs or characteristics that might provide clues about their role in venom function (*e.g.*, cysteine-rich), but found none (Figure 1B).

Proteomic analyses help confirm the subset of venom gland transcripts that are expressed as functional components in venom. SDS-PAGE separation of our *P. tristis* crude venom prior to proteomic analysis shows two abundantly expressed groups of peptides and proteins: one between 20–25kDa and the other <~12kDa in size (Figure 2A). LC-MS/MS analysis after trypsin digestion identified 401 peptides from mass spectra with significant matches (≥96%) to peptides and proteins translated from our transcriptome data across three major groups including seven astacins, six venom peptide groups, and one protein of unknown function (Figure 2B). We are confident in the identification of proteomic components, however lack of detection may be false negatives and cannot be interpreted that a transcript is not a venom component. Previous proteomic analysis of plectreurid venom has focused on detection and analysis of acylpolyamines and venom peptides <10kDa (Branton et al, 1987, 1993; Quistad et al, 1993; Quistad and Skinner, 1994;

Zhou et al, 2013). Our data indicate that astacins and small peptides with classic motifs of neurotoxins are abundant and diverse in *Plectreurys* venom (Figures 1C and 2B). Below, we describe the protein and polypeptide components found in the transcriptome of *P. tristis* and discuss evidence of detection in crude venom.

Toxic venom proteins

Astacin metalloproteases are the only proteins that are known venom toxins we identified in the venome of *P. tristis*, making up 5% of the transcriptome (11 cDNAs) and a majority of the proteome (249 peptide matches) (Figures 1, 2). This set of homologous proteins is variable, with the predicted molecular weight (MW) of pro-enzyme sequences ranging from 23–26.6kDa, matching a densely staining region of proteins in the SDS-PAGE gel (Figure 2A). The most abundant astacin transcript, cDNA 589, is also the largest (26.6kDa), and represents 26% of the astacin peptides detected in the proteome (Figure 2B). Astacins are a family in the metzincin clan of metallopeptidase zymogens that become proteolytic upon activation (reviews in Gomis-Rüth et al, 2012 and Cerdà-Costa and Gomis-Rüth, 2014). Astacins were first characterized as toxic spider venom components in *Loxosceles* by da Silveira et al (2007), and the gene family was further described by Trevisan-Silva et al (2010) who identified multiple LALPs (*Loxosceles* astacin-like proteases) from *L. intermedia, L. gaucho* and *L. laeta.* In *Loxosceles* venoms, astacins degrade matrix proteins (gelatin, fibronectin and enactin) suggesting a key role in the spreading of venom components (da Silveira et al, 2007), and different paralogs from the same species vary in specificity of gelatinolytic function (Trevisan-Silva et al, 2013). The diversity of astacins in *P. tristis* likely also includes a range of functional specificities.

Astacin cDNAs from *Plectreurys* had significant hits in public databases (e10^{-7}–e10^{-33}), sharing up to 47% identity with a Zn-metalloprotease from the predatory mite, *Metaseiulus occidentalis*, but only 39% with astacins reported from *Loxosceles*. Even stronger hits were detected within Haplogynes where matches are as high as e10^{-44} with *Sicarius* and e10^{-48} with *Physocyclus* astacins (unpublished). Figure 3 shows an alignment of full-length pro-enzyme transcripts we isolated, where proteomic peptides are well represented across the length of the sequences (underlined). The translated sequences contain highly conserved residues and motifs (boxed) characteristic of astacin metalloproteases, including: the aspartate switch region motif (**FXGD**) in the proenzyme sequence, conserved cysteine residues, the Zn-binding and protease domain (**HEXXHXXGXXHE**) and the conserved Met-turn motif (**SXMXY**) (Guevara et al, 2010; Gomis-Rüth et al, 2012; Cerdà-Costa and Gomis-Rüth, 2014). There is considerable variation among homologous sequences, including large indels (>20aa), both within *Plectreurys* and among arachnids we hit in databases, rendering alignments across the full length of the protein ambiguous. However, the active sites described above are largely conserved among *P. tristis* paralogs and across a broad phylogenetic range of taxa. Interestingly, phylogenies including all published sequences with hits <e10^{-5} support *Plectreurys* astacin sequences as monophyletic and more closely related to tick astacin sequences than any others in our dataset including *Loxosceles* (data not shown). We are
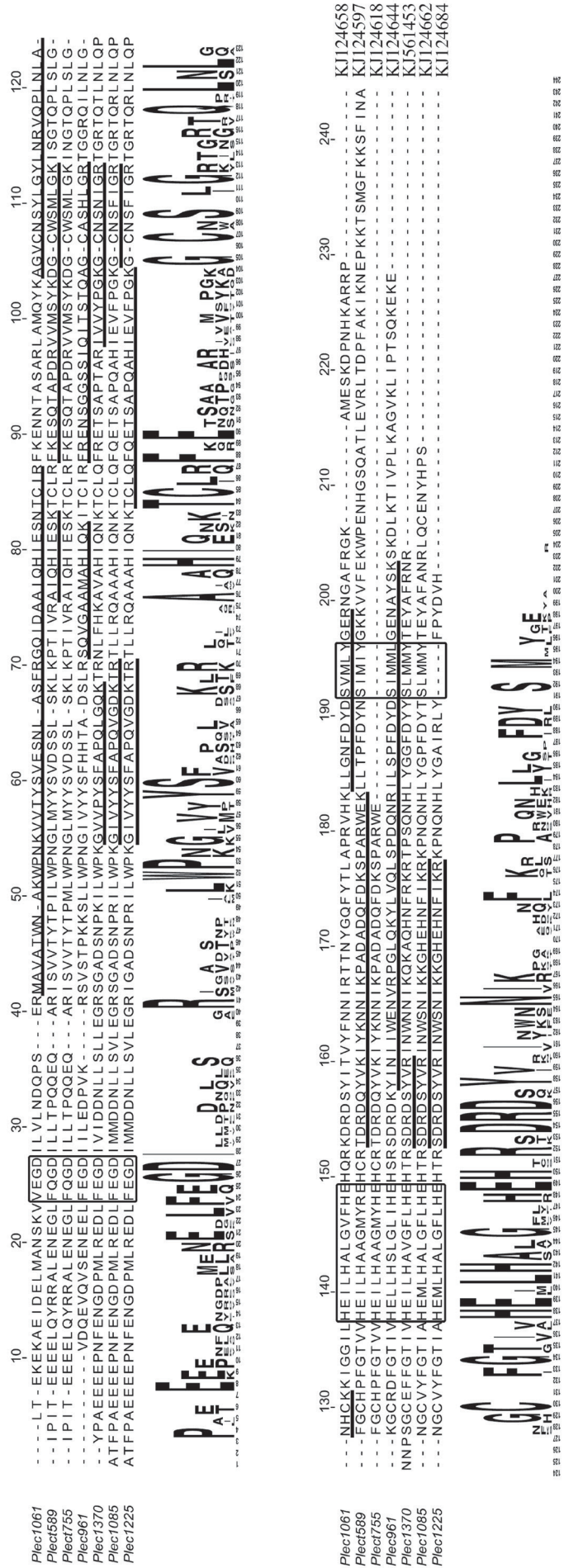
Figure 3. Astacin sequence analysis. An alignment of nonredundant *Plectreurys* pro-enzyme astacins is shown (signal sequence is omitted). Important conserved astacin motifs are boxed. Peptides detected in the proteomic analysis are underlined. The consensus logo (below) was generated using WebLogo (http://weblogo.berkeley.edu) of a ClustalO amino acid alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/), where the tallest letters represent the most conserved amino acids across all seven sequences. Corresponding GenBank accession numbers are listed after the C-terminus of each protein sequence.

pursuing a more detailed analysis of the evolution of this gene family within haplogyne spiders.

Candidate venom peptide toxins
Peptides with characteristics that are typical of peptide toxins constitute 9% of the *P. tristis* transcriptome (Figure 1A) and 32% of the proteome (Figure 1C, Figure 2). We identified 19 cDNAs all of which had significant hits to toxic venom peptides in ArachnoServer (Table 1). All sequences we identified as venom peptides in this study translate into polypeptides that contain posttranslational processing sites for signal and/or mature cleavage. Mature peptide sequences are rich in cysteine residues with characteristic patterns for forming disulfide bonds to fold into compact structures (Table 1). Predicted sizes of mature venom peptides presented in Table 1 range from ~4–12kDa; however, the native sizes in venom may vary due to posttranslational modification and/or C-terminal processing. Figure 2 shows that *P. tristis* has an abundant set of venom peptides that migrate within a range of ~8–12kDa.

Within the set of 19 putative peptide toxin transcripts, we identify eight distinct groups of toxins in the transcriptome (Table 1, Figure 4) six of which are new to this work. We name the toxins using the reference nomenclature plectoxins (PLTX) (King et al, 2008). To our knowledge, ten venom peptides in five groups have been previously identified from *P. tristis* venom (Figure 4A). We identified representatives of one previously known toxin in the proteome (U_3-PLTX-Pt1a) and one other (U_1-PLTX-Pt1a) in the transcriptome. Panels B and C in Figure 4 show alignments of all plectoxins, divided based on their best database hits, which overlap. The transcripts we identify expand two previously known groups of toxins (U_1-PLTX and ω-PLTX) and add six new groups, U_4- through U_9-PLTX (Table 1). We designate sequences as paralogs by the amount of sequence divergence (more than a few amino acids differ) in an alignment and evidence of them being in different phylogenetic clades. We estimate sequences to be allelic variants when they differ by one or two amino acids and are in a single phylogenetic clade with little divergence. Within the largest previously known group, U_1-PLTX (Table 1, Figure 4A), many sequences have multiple allelic variants that are identical in the mature peptide, but differ in the signal peptide. We identified one of the seven sequences of U_1-PLTX-Pt1a that is identical to the signal peptide variant labeled U_1-PLTX-Pt1a_6. We also identified a peptide that aligned well with U_1-PLTX sequences, but differed in the mature peptide enough to be a distinct paralog we named U_1-PLTX-Pt1g (Figure 4). New groups are designated when alignments show marked differences due to insertions, deletions or enough amino acid changes that affect the cysteine skeleton patterns (e.g. U_5-U_9-PLTX).

All peptide groups have cysteine skeleton patterns (-C-C-CC-C-C-) characteristic of knottin or inhibitor cysteine knot (ICK) peptides, including principle structural motifs (PSMs) –CXC-CC- and extra structural motifs (ESMs) –C1C- found in many spider toxins (Kozlov et al, 2005) (Table 1). Mature sequences in ω-, U_1-, U_4-, U_6- and U_7-PLTX groups are predicted to be knottins by the Knoter1d program (brackets, Figure 4B, 4C). The compact structure of these peptides provides great stability and proteolytic resistance.

Their high affinity and distinct selectivity for manipulating ion channels makes them attractive targets for discovery of toxins with potential utility as drugs or insecticides (Vassilevski et al, 2009; Saez et al, 2010; King, 2011; Smith et al, 2013).

The cDNA sequence we identify as ω-PLTX-Pt1b is the most abundant peptide detected in the proteome (15%). Its mature sequence shares 95% identity with ω-PLTX-Pt1a and likely has the same molecular target(s). While the function of most of these peptides is unknown, ω-PLTX-Pt1a (originally identified as alpha-PLTX-II (Branton et al, 1987)) has been extensively studied. It acts to inhibit presynaptic Ca^{2+} channels in *Drosophila* (Branton et al, 1987; Leung et al, 1989) and also in mammals (Feigenbaum et al, 1988). It can allosterically inhibit ω-conotoxin from binding to its target (Lundy and Frew, 1993) and was shown not to affect glutamate receptors (Jackson et al, 1987). Zhou et al (2013) recently identified δ/ω-PLTX-Pt1a which affects both Na^+ and Ca^{2+} channels in neuromuscular junctions of *Drosophila*.

A notable feature of the ω-, and δ/ω-PLTX is that they are posttranslationally modified. ω-PLTX-Pt1a has an *O*-linked palmitoyl moiety on the threonine residue near the C-terminus (circled in Figure 4B); cleavage of this fatty acylation results in decreased potency by at least two orders of magnitude (Branton et al, 1993; Bódi et al, 1995). ω-PLTX-Pt1b has a homologous threonine residue positioned near the C-terminus (dashed circle, Figure 4B), a likely candidate for posttranslational modification. δ/ω-PLTX-Pt1a (Zhou et al, 2013) contains an *O*-palmitoyl modification of a serine residue near the C-terminus (circled in Figure 4B), which is required for function. Interestingly, direct searches in our proteomics dataset found no evidence of palmitoylation. Nevertheless, these unique site modifications may play important roles including aid in targeting and tethering to the membrane while the peptide carries out its neurotoxic function on ion channel proteins (Branton et al, 1993; Zhou et al, 2013).

Close inspection of the larger U_8- and U_9-PLTX groups shows that the mature sequence is the only region of detectable homology to sequences in databases (Figure 4C), and each of these six sequences contains a long, polar extension adding 2- and 4kDa, respectively, after the C-terminal cysteine residue (underlined, Table 1). The C-terminal stretch of amino acids after the final cysteine of spider venom peptides is not unique to plectoxins; many lycotoxins from *Lycosa singoriensis* and some theraphotoxins from *Chilobrachys jingzhao* also have >20aa (ArachnoServer), without patterns indicative of secondary structures or posttranslational processing motifs that may give clues to any role in the peptide's function.

Other proteins
Twenty percent (45 cDNAs) of our transcriptome sequences are classified as "other proteins" (Figure 1A), which include sequences with open reading frames (ORFs). Six groups ("entities") confidently hit cDNA sequences with unknown function in NCBI nr or EST databases (e≤10^{-5}). Four of the six unknowns hit "secreted" or "salivary" peptides and proteins from ticks, but a majority (32 full length entities) do not

A.

NCBI accession #	plectoxin	detected in transcriptome	detected in proteome	MW (kDa)	pI	reference
P34079	ω-PLTX-Pt1a			4.90	5.5	Branton et al. (1987, 1993)
KJ124719	ω-PLTX-Pt1b	X	X	4.00	9.2	this study
n/a	δ/ω-PLTX-Pt1a			4.80	8.6	Zhou et al. (2013)
P36983	U$_1$-PLTX-Pt1a	X		5.60	7.7	Quistad & Skinner (1994); Leisy et al. (1996)
P36984	U$_1$-PLTX-Pt1b			6.90	5.2	Quistad & Skinner (1994); Leisy et al. (1996)
P36985	U$_1$-PLTX-Pt1c			8.90	5.2	Quistad & Skinner (1994); Leisy et al. (1996)
P36986	U$_1$-PLTX-Pt1d			5.00	5.0	Quistad & Skinner (1994)
P36987	U$_1$-PLTX-Pt1e			partial	n/a	Quistad & Skinner (1994)
P36988	U$_1$-PLTX-Pt1f			5.00	5.6	Quistad & Skinner (1994)
KJ124642	U$_1$-PLTX-Pt1g	X	X	5.10	7.7	this study
P36989	U$_2$-PLTX-Pt1a			5.20	9.1	Quistad & Skinner (1994)
P36990	U$_3$-PLTX-Pt1a		X	5.10	9.1	Quistad & Skinner (1994)
KJ124621	U$_4$-PLTX-Pt1a	X		4.00	9.2	this study
KJ124657	U$_5$-PLTX-Pt1a	X	X	7.30	5.6	this study
KJ124651	U$_6$-PLTX-Pt1a	X	X	8.10	6.3	this study
KJ124616	U$_7$-PLTX-Pt1a	X	X	8.30	8.6	this study
KJ124648	U$_7$-PLTX-Pt1b	X	X	8.30	8.6	this study
KJ124608	U$_8$-PLTX-Pt1a	X		10.50	8.1	this study
KJ124641	U$_8$-PLTX-Pt1b	X		10.50	8.1	this study
KJ124598	U$_9$-PLTX-Pt1a	X		11.50	8.6	this study
KJ124609	U$_9$-PLTX-Pt1b	X		11.70	8.6	this study
KJ124633	U$_9$-PLTX-Pt1c	X		11.60	8.4	this study

B. (Amino acid sequence alignment; sequences listed: U$_4$-PLTX-Pt1a, Γ-CNTX-Pn1a, κ-CNTX-Pn1a, ω-CNTX-Pn1a, U$_3$-PLTX-Pt1a, U$_{16}$-LCTX-Ls1b, U$_2$-PLTX-Pt1a, U$_6$-PLTX-Pt1a, δ/ω-PLTX-Pt1a, U$_1$-PLTX-Pt1g, U$_1$-PLTX-Pt1c, U$_1$-PLTX-Pt1a_6, U$_1$-PLTX-Pt1d, U$_1$-PLTX-Pt1f, U$_1$-PLTX-Pt1b, μ-HXTX-Mg2a, ω-PLTX-Pt1b, ω-PLTX-Pt1a)

C. (Amino acid sequence alignment; sequences listed: U$_3$-AATX-Ce1a, U$_{33}$-TRTX-Cj1a, U$_7$-PLTX-Pt1a, U$_7$-PLTX-Pt1b, U$_1$-HXTX-Iw1e, U$_1$-HXTX-Hi1a, U$_9$-TRTX-Hh1a, U$_{19}$-CNTX-Pn1a, U$_5$-PLTX-Pt1a, U$_8$-PLTX-Pt1b_1, U$_8$-PLTX-Pt1a, U$_8$-PLTX-Pt1b, U$_9$-PLTX-Pt1a, U$_9$-PLTX-Pt1b, U$_9$-PLTX-Pt1c)
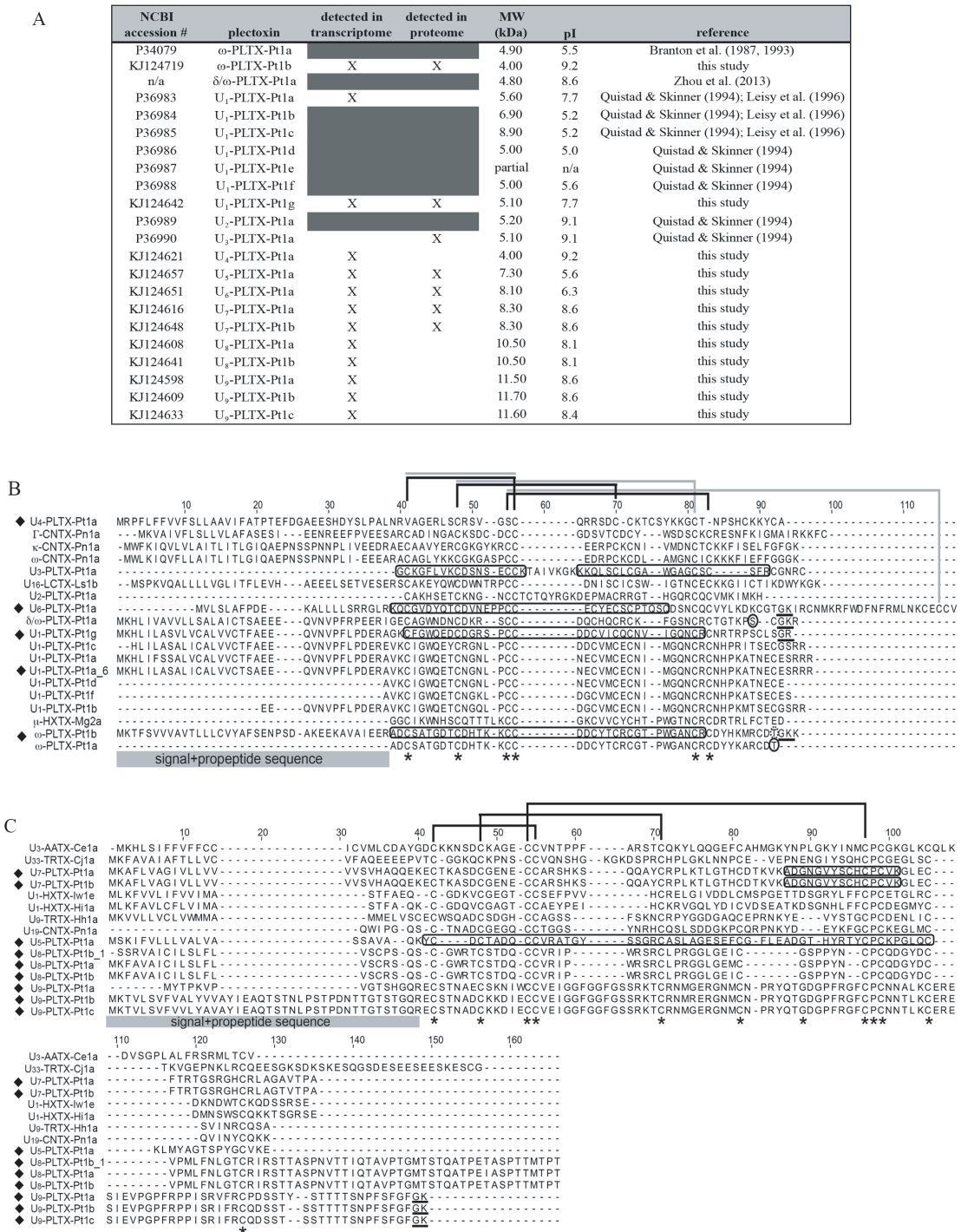
Figure 4. Summary of plectoxins (PLTX). **A.** All plectoxins identified to date are listed. Black boxes represent known peptides that were not identified in either transcriptomic or proteomic methods in this study. Amino acid alignments of venom peptide sequences were divided into two groups (**B**) and (**C**) that each unite sets of sequences with overlapping database hits. Peptides identified in this study have a rhombus (◆) to the left of the sequence name. Predicted signal and propeptide sequences are underlined in gray. Cysteines in the mature sequence have an asterisk (*). Peptides detected in the proteome are boxed. Black and gray brackets above sequences indicate predicted disulfide bonds forming an ICK peptide based on analysis using Knoter1d. Homologous sequences are listed according to ArachnoServer nomenclature as plectoxins (PLTX), ctenitoxins (CNTX), lycotoxins (LCTX), hexatoxins (HXTX), aranetoxins (AATX), and theraphotoxins (TRTX). Circled amino acids in (**B**) contain an *O*-palmitoyl moiety; the dashed circle represents a likely *O*-palmitoylation site. Underlined amino acids are potential recognition sites for C-terminal modification.

hit anything using searches of public nucleotide databases. Half of these sequences share homology with sequences we have identified in our Haplogyne database ($e10^{-5}$–$e10^{-10}$). Only one of these sequences was detected in the proteome (cDNA 925), making up 6% of the crude venom (Figures 1C and 2B). We found two identical transcripts for this protein, but neither were full length (no Methionine), so we cannot predict how much larger than 15kDa it may be in native

Table 2. Paralytic doses (PD$_{50}$ values) of assayed spider venoms and peptides. PD$_{50}$ or ED$_{50}$ values are listed from collections of bioassays testing spider venom or individual venom peptides on lepidopteran larvae or crickets. For comparative purposes, units from Magi peptides were converted to µg/g, using the average oxidized mass for calculation (ArachnoServer). PLTX peptides with an asterisk (*) have identical primary structures, but native polypeptides were shown to elute from HPLC with different profiles (Quistad and Skinner, 1994).

Venom source Purified peptide	PD$_{50}$ or ED$_{50}$ (µg/gm)	Organism assayed	Reference
Plectreurys tristis - crude	3.3	*Acheta domestica* (cricket)	this study
P. tristis - crude	0.03 µl/gm	*Manduca sexta* (tobacco hornworm)	Quistad and Skinner (1994)
	0.07 µl/gm	*Heliothis virescens* (tobacco budworm)	
	0.06 µl/gm	*Spodoptera exigua* (beet armyworm)	
Plt-V (U$_1$PLTX-Pt1a)*	0.04	*M. sexta*	
	0.52	*H. virescens*	
	0.44	*S. exigua*	
Plt-VI (U$_1$-PLTX-Pt1a)*	0.10	*M. sexta*	
	0.21	*H. virescens*	
	0.32	*S. exigua*	
Plt-VIII (U$_1$-PLTX-Pt1b)	0.42	*M. sexta*	
	1.89	*H. virescens*	
	>5	*S. exigua*	
Plt-IX (U$_2$-PLTX-Pt1a)	>11	*M. sexta*	
	>11	*H. virescens*	
Plt-X (U$_3$-PLTX-Pt1a)	>10	*M. sexta*	
	>10	*H. virescens*	
	13	*S. exigua*	
Plt-XI (U$_1$-PLTX-Pt1c)	0.36	*M. sexta*	
	1.38	*H. virescens*	
	0.24	*S. exigua*	
Loxosceles arizonica - crude	2.6		Zobel-Thropp et al (2010)
Sicarius cf. damarensis - crude	0.4	*A. domestica*	
S. cf. hahni - crude	1.5		
S. rugosa - crude	3.2		
Phoneutria nigriventer - crude	43.5	*Gryllus assimilis* (cricket)	Manzoli-Palma et al (2003)
Nephilengys cruentata - crude	24.6		
Acanthoscurria paulensis			Mourão et al (2013)
Ap1a	13	*S. frugiperda* (fall armyworm)	
Macrothele gigas			Corzo et al (2003)
Magi-1 (µ-HXTX-Mg1b)	>150.7	*S. litura* (tobacco cutworm)	
Magi-2 (µ-HXTX-Mg1a)	87		
Magi-3 (µ-HXTX-Mg2a)	53.8		
Magi-4 (δ-HXTX-Mg1a)	6.2		
Magi-5 (β-HXTX-Mg1a)	28.3		
Magi-6 (U$_7$-HXTX-Mg1a)	12.5		
Apomastus schlingeri - crude	0.01µl/gm	*M. sexta*	Skinner et al (1992)
ApsI (U$_1$-CUTX-As1a)	1.3		
ApsII	0.6		
ApsIII (µ-CUTX-As1a)	>5		
ApsIV (U$_1$-CUTX-As1b)	0.5		
ApsV	0.3		
ApsVI (U$_1$-CUTX-As1c)	0.1		
ApsVII (U$_3$-CUTX-As1a)	>5		
ApsVIII	0.5		
ApsIX (U$_1$-CUTX-As1d)	0.4		

form. Finally, 32 full-length cDNA sequences are unique to *Plectreurys* in that they don't have any matches $\leq 10^{-5}$ against any databases we searched. Sequences range from 34–153aa with predicted molecular weights of polypeptides ranging from 2.6–15.7kDa. None of these were detected in the proteome.

Neurotoxicity of *P. tristis* venom

With the goal of placing this venomic analysis in the context of venom function, we assayed the potency of whole crude *Plectreurys* venom in crickets. The PD_{50} one hour after injection was 3.3μg/gm cricket. We obtained an average of 51μg of total protein per spider, which is likely an underestimate of the total amount of venom in an individual, but is over 15x the estimated paralytic dose. While we did not quantify LD_{50}, over half of the individuals were dead after 24 hours at our highest dose (1.6μg) and none of the animals recovered from loss of righting response. The only previous analysis of *Plectreurys* potency is from individual peptides isolated from crude venom that were injected into lepidopteran larvae (Quistad and Skinner, 1994). U_1-PLTX-Pt1a was the most insecticidally potent venom peptide. Interestingly, in previous work, U_3-PLTX-Pt1a (Plt-X) was the most abundant peptide in *P. tristis* venom and among the least potent (Quistad and Skinner, 1994); it was the second most abundant peptide identified in our proteome analysis (Figure 2B). ω-PLTX-Pt1a was not identified or assayed in that study.

Comparing our *P. tristis* PD_{50} value for crude venom with those from Quistad and Skinner (1994) and Skinner et al (1992) is challenging because their values are provided in μl/gm units and we are uncertain of the quantity of total protein in the venom assayed. In fact, comparing potencies based on bioassays is generally challenged by differences in injection methods, target organisms and different scoring methods among researchers. We can, however, compare our data to that from other spider venoms where the relative units are the same. Table 2 is a summary of paralytic and effective doses (PD_{50} or ED_{50}) accumulated from bioassays using spider venoms on various target species with units converted to μg/gm when possible for comparison. When using crickets for bioassays, *Plectreurys* venom potency (3.3μg/gm) is about the same as venom from the Central American sicariid spider *Sicarius rugosa* (3.2μg/gm) and nearly as potent as *Loxosceles arizonica* (2.6μg/gm). It is up to 7x less potent than venom from African *Sicarius* species (0.4 and 1.5μg/gm) and 8–13x more potent than *Nephilengys cruentata* (24.6μg/gm) and *Phoneutria nigiventer* (43.5μg/gm) spiders. When comparing results from lepidopteran injections, *Plectreurys* venom is much more potent than any of the individual hexatoxins (HXTX) from the hexathelid *Macrothele gigas* (6–151μg/gm) and Ap1a from *Acanthoscurria paulensis* (13μg/gm) and much less potent – up to 33x less – than cyrtautoxins (CUTX) from *Apomastus schlingeri*. Although *Plectreurys* venom is not known to be dangerous to humans, its components can be quite toxic to agricultural pests.

While assaying crickets for venom potency, we recorded visibly detectable effects of venom on cricket movement. At each dose, there was a notable change in the cricket's neuromuscular response as compared to those designated as controls (injected with saline). As venom dosage increased, the amount of uncontrollable twitching increased as well. Constant twitching was most evident in terminal portions of all legs, particularly the tarsal regions, and also in both antennae; all responses occurred independently. At higher doses, muscles in the ventral side of the abdomen oscillated in waves. The neurotoxins in *Plectreurys* venom at these doses do not appear to be lethal, since all crickets were alive – and some still twitching - 24hr after injection. This is in stark contrast to what we observed with cricket injections using venom from *L. arizonica* (Zobel-Thropp et al, 2012). It will be informative to test individual neurotoxins from *Plectreurys* venom on crickets and identify specific neurophysiological targets.

CONCLUSIONS

Our analysis of the proteome and transcriptome from *P. tristis* venom gland tissue is a first pass contributing to the full set of components of the venome of these spiders. We have expanded the previously known set of venom peptides and have identified additional components through isolation and sequence analysis of proteins. We provide evidence that astacins are a relatively abundant family of toxins in the venom, and the presence of numerous homologs suggests that this gene family has likely evolved to play an important role in venom function. The phylogenetic position of *Plectreurys* makes the discovery of this set of toxins useful for comparative understanding of venom toxin distribution and diversity among spiders.

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COMPETING INTERESTS

None declared.

ABBREVIATIONS

SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis
LC-MS/MS; liquid chromatography-mass spectrometry
HPLC; high pressure liquid chromatography

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