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Comparative analyses of venoms from American and African *Sicarius* spiders that differ in sphingomyelinase D activity

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ABSTRACT

Spider venoms are cocktails of toxic proteins and peptides, whose composition varies at many levels. Understanding patterns of variation in chemistry and bioactivity is fundamental for understanding factors influencing variation. The venom toxin sphingomyelinase D (SMase D) in sicariid spider venom (*Loxosceles* and *Sicarius*) causes dermonecrotic lesions in mammals. Multiple forms of venom-expressed genes with homology to SMase D are expressed in venoms of both genera. SMase D activity levels differ among major clades with American *Sicarius* vastly reduced relative to all *Loxosceles* and African *Sicarius* despite expression of SMase D homologs in venoms of American *Sicarius*. Here we report comparative analyses of protein composition and insecticidal activity of crude venoms from three *Sicarius* species, two from South Africa and one from Central America. Comparative 2-dimensional electrophoresis shows dense regions of proteins in the size range of SMase D in all three species, but there are differences in sizes and isoelectric points (pIs). Few proteins strictly co-migrate and there are clusters of proteins with similar pIs and molecular weights whose patterns of similarity do not necessarily reflect phylogenetic relatedness. In addition, PD_{50} estimates on crickets indicate a small though significant decrease in potency of South American *Sicarius* venoms relative to African species.

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1. Introduction

Venoms are complex cocktails of proteins, toxins and low molecular weight components. In multiple venomous lineages (snakes, scorpions, cone snails and spiders), differences in the composition of extruded venom occur among related species, between sexes within species, and ontogenetically within individuals (McCrone and Netzloff, 1965; Atkinson, 1981; Atkinson and Walker, 1985; Müller et al., 1989, 1992; Chippaux et al., 1991; Daltry et al., 1996; de Oliveira et al., 1999, 2005; Binford, 2001a; Young and Pincus, 2001; Escoubas et al., 2002; Herzig et al., 2002, 2008; Rash and Hodgson, 2002; Richardson et al., 2006; Batista et al., 2007; Birrell et al., 2007). Comparative analyses suggest that venom variation may correlate with geography, habitat, and prey capture strategies (*e.g.* Binford, 2001b; Remigio and Duda, 2008); however there is much to learn about factors that influence variation, and the mechanisms by which it is generated.

The complex of toxins in an individual's venom at a given time is influenced by the expression of different members of large gene families (for reviews, see Kordis and Gubensek, 2000; Zhang, 2003; Rodríguez de la Vega and Possani, 2004, 2005; Calvete et al., 2005; Escoubas, 2006; Fry et al., 2008) and posttranslational protein modification (Buczek et al., 2005; Escoubas, 2006; Earl et al., 2006; Birrell et al., 2007; Rehana and Kini, 2007; Fry et al., 2008). The combination of these processes leads to multiple paralogs and isoforms of bioactive components in

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individual venoms. Evolution of gene families, expression patterns and posttranslational modifications can lead to substantial differences among taxa (recent papers from Buczek et al., 2005; Escoubas, 2006; Earl et al., 2006; Birrell et al., 2007; Rehana and Kini, 2007; Fry et al., 2008; Binford et al., 2009).

For medically relevant venom chemicals, studying patterns of variation and factors that influence them is helpful for understanding the range of risks associated with envenomation and possible broad-range treatments. Venom from spiders in the family Sicariidae including *Loxosceles* (violin or fiddleback spiders) and *Sicarius* (six-eyed sand spiders) can cause dermonecrotic lesions in humans (*Loxosceles* reviews in da Silva et al., 2004; Vetter, 2008; *Sicarius* in Newlands and Atkinson, 1988; van Aswegen et al., 1997). A family of enzymes that includes sphingomyelinase D (SMase D) is the main culprit. Over the years, the purified protein has been shown to trigger a cascade of physiological events that lead to dermonecrosis (Geren et al., 1976; Kurpiewski et al., 1981; Barbaro et al., 1996, 2005; Tambourgi et al., 1998, 2002, 2004; Veiga et al., 2000, 2001; Fernandes-Pedrosa et al., 2002; Cunha et al., 2003; Ramos-Cerrillo et al., 2004; de Oliveira et al., 2005; Pretel et al., 2005; da Silveira et al., 2006, 2007; Chaim et al., 2006; Felicori et al., 2006; Ribeiro et al., 2007; Appel et al., 2008). SMase D belongs to a multi-gene family recently named *SicTox*, multiple members of which are expressed in venom glands of both *Loxosceles* and *Sicarius* (Binford et al., 2009 and references therein). *SicTox* genes have recently been shown to be the most abundant known transcript in venom gland tissue of *Loxosceles laeta* (Fernandes-Pedrosa et al., 2008).

Of the sicariid species much less is known about the venoms and biology of *Sicarius* than *Loxosceles*. The genus *Sicarius* is only found in Southern Africa and dry regions of South and Central America. African and American species are reciprocally monophyletic (Binford et al., 2008). *Sicarius* are generally much larger than *Loxosceles* and some species produce an order of magnitude more venom protein per individual than the average *Loxosceles* species (Binford and Wells, 2003). While sicariid venom, particularly SMase D in *Loxosceles*, has received a lot of attention because of its effects on mammals, there has been little focus on venom diversity in this lineage as a function of its natural role in prey capture. All known *Sicarius* live in dry habitats in or near sandy areas where they cover themselves and their egg sacs with sand particles (Levi and Levi, 1969; Duncan et al., 2007). Data for natural prey of *Sicarius* are limited but across species we have observed *Sicarius* in the field in both Africa and America eating cockroaches, ants, crickets, scorpions and spiders (personal observations) and have seen no evidence of consumption of vertebrates. There is also no evidence of *Loxosceles* consuming vertebrates (Hite et al., 1966; Fischer and Vasconcellos-Neto, 2005; pers obs). Diet breadth for any single species of *Sicarius* is not known, but we have not seen any evidence of specialization in our anecdotal observations in the field or laboratory. *Sicarius* capture prey by pulling them to their jaws, biting and holding on while prey are rapidly immobilized (pers obs).

The only known venom components in *Sicarius* venoms are proteins homologous to SMase D (Binford et al., 2009).

Many other venom components are known in *Loxosceles* (review in da Silva et al., 2004) and, given their close relationship with *Sicarius* and similarities in 1-D venom separation patterns (Binford and Wells, 2003; Binford et al., 2009), some subset of toxins in *Loxosceles* venoms are likely to have homologs expressed in the later genus. Of particular note with respect to bioactivity on natural arthropod prey, genes with homology to insecticidal toxins have been isolated from venoms of *Loxosceles intermedia* (de Castro et al., 2004) and *L. laeta* (Fernandes-Pedrosa et al., 2008).

The abundance of SMase D and homologous proteins in venoms is consistent with an important role in prey immobilization, but this role has not been studied. We have recently detected striking differences in SMase D activity between African and American *Sicarius*. We compared crude venoms from 14 *Sicarius* populations that represent a large breadth of phylogenetic diversity including at least five species from South Africa and Namibia, and five species from the Americas (Argentina, Peru, Central America). Strikingly, all venoms from African species had high levels of SMase D enzyme activity whereas all American species had three orders of magnitude lower activity than African species (Binford et al., 2009).

The differences in SMase D activity between African and American *Sicarius* have inspired us to compare patterns of variation in expressed venoms and bioactivity in natural prey. Specifically, we compare crude venoms from 3 species of *Sicarius*, two from Africa and one from Central America. *Sicarius rugosa* is found in tropical dry forests and desert rocky outcrops with a range that minimally includes Costa Rica and El Salvador. *S. cf. damarensis* is from a population in the Northern Cape Province of South Africa in a dry gorge in a transitional area between the fynbos and Karoo biomes. *S. cf. hahni* is from an area of sandy outcrops in forested mountains of the northern Drakensberg Mountains in South Africa's Northern Province. We compare venoms from these species by (1) assaying SMase D activity across a range of venom concentrations, (2) comparing protein profiles using 2-dimensional separations and (3) testing whether these venoms differ in doses required to paralyze crickets.

2. Materials and methods

2.1. Taxon inclusion

The spiders used in this work were collected by Greta Binford (GJB) and colleagues in the field as mature females. We compared venoms of mature female spiders of *S. rugosa* (Palo Verde, Costa Rica, N 10°20.836, W 085°20.467); *S. cf. damarensis* (referred to as *Sicarius testaceus* in Binford and Wells (2003)) from Oorlogskloof, South Africa (S 31°29.590, E 19°06.552) and *S. cf. hahni* (referred to as *Sicarius hahni* in Binford and Wells (2003)) from the Strydpoort Mountains in the Northern Province of South Africa (S 24°09.820, E 29°55.835). *Sicarius* species nomenclature is challenging and in flux. The names here reflect the most accurate identifications we can currently make. The addition of "*cf.*" to the species name is standard taxonomic nomenclature that indicates that characteristics of individuals were consistent with, but differed slightly from

characteristics in formal species definitions for these species (see Binford et al., 2008 for further details). Further details of collecting localities and vouchers are available from GJB by request. Spiders were maintained in the laboratory as in Binford et al. (2009).

2.2. Venom source

We used electrical stimulation to extract venom from all spiders as described in Binford and Wells (2003). To standardize for degree of replenishment of proteins in the venom reservoir we collected venom two weeks after feeding and no less than two months after a previous venom collection. We pooled venoms among individuals to standardize for possible differences due to sex, season or maturity. We only used mature female individuals collected on the same day from single localities. We used venom pools from 4 to 7 individuals for the SMase D activity assays, except *Loxosceles arizonica*, which was from a pool of 15. For 2-D electrophoresis, the venom pools included 6, 2 and 7 individuals for *S. cf. damarensis*, *S. cf. hahni* and *S. rugosa* respectively. The smaller number of individuals in the venom pool of *S. cf. hahni* reflects the fact that we had only 2 mature females from that population at that time. We have not previously observed variation among individuals within the same sex, season and population in 1-D electrophoretic separations (unpublished data) and were therefore not concerned with individual differences that might result from different numbers of individuals included in venom pools. The pools of venom used for insecticidal activity assays ranged between 2 and 4 individuals.

2.3. SMase D activity assay

We assayed sphingomyelinase D activity using a modification of the Amplex™ Red Phospholipase D Assay Kit (Molecular Probes), as described in Binford and Wells (2003) and Binford et al. (2009). In addition to *Sicarius*, we included samples from *L. arizonica* for comparison with a species with well established SMase D activity in venom, and *Drymusa dinora* for comparison with a closely related genus that does not have proteins in the size range of SMase D (Binford and Wells, 2003) and screening for venom-expressed *SicTox* genes has never yielded genes homologous to SMase D (Binford et al., 2009). Fluorescence intensity (proportional to SMase D activity) was measured from reactions that contained 0.5 or 1.0 μg of crude venom and 10 or 50 μg of venom from species with low activity as compared to the blank. A *Drymusa* sample at 50 μg was not analyzed because of low venom availability. Buffer was substituted for venom in blank samples.

2.4. SDS-PAGE

We separated crude venom proteins (7 μg) using 1-dimensional SDS-PAGE (12%). We loaded a broad range molecular weight standard (New England Biolabs, P7702S) for size reference and visualized proteins using a standard silver stain protocol.

We compared venoms among species using 2-D Differential In-Gel Electrophoresis (DIGE) SDS-PAGE in the Proteomics Lab at the University of Arizona. This method simultaneously separates multiple crude protein mixes on the same gel. Each sample is uniquely stained with a fluorescent dye, enabling separation of proteins with internal standardization. We diluted 50 μg of crude venom protein in 30 mM Tris, pH 8.5, 3 M urea and 4% CHAPS. We used 1 μL of a unique CyDye™ color (Amersham Biosciences) to distinguish each sample. To separate proteins horizontally by isoelectric focusing, we loaded combined samples on a 24 cm DryStrip in a voltage box (Amersham Biosciences) at 8000 V for 8 h and 20 min (66 000 V h total). To separate by size in the second dimension, we used 15% tris-tricine polyacrylamide gels and ran the samples at 30 V for 6 h and 40 min, 500 V for 1 h, and 8000 V for 8 h and 20 min (68,340 V h total). We imaged each CyDye™ with the appropriate laser excitation wavelength and used the Typhoon Imager software program (Amersham Biosciences) to standardize and determine the intensity of each emission.

2.5. Paralytic dose (PD$_{50}$) estimation

To investigate differences in potency on prey among crude venoms of *Sicarius* species that differ in SMase D activity, we quantified the dose (absolute μg and μg/g cricket) at which 50% of crickets (*Acheta domestica*) injected were paralyzed after 60 min (PD$_{50}$). We diluted each venom sample in physiologically buffered lepidopteran saline solution (5 mM KH$_2$PO$_4$/100 mM KCl/4 mM NaCl/15 mM MgCl$_2$/2 mM CaCl$_2$, pH 6.5) and established a dosage range distributed around the PD$_{50}$ by performing preliminary injections. Experimental doses (total μg crude venom protein) were 0.07, 0.14, 0.21, 0.28 for *S. cf. damarensis;* 0.13, 0.26, 0.39, 0.52 for *S. cf. hahni;* and 0.18, 0.36, 0.54, 0.70 for *S. rugosa*. We did three replicate assays for each species. For each replicate we assayed 20 crickets per dose by injection in the dorsal mesothorax using a PB-600 dispenser with a gastight syringe (Hamilton Co., Reno, NV). With each replicate, we injected an additional 20 control crickets with 0.8μl of physiologically buffered lepidopteran saline, a volume equal to the largest dose volume. We scored paralysis every 10 min for 1 h based on the ability of a cricket to right itself. We did not score death at 60 min, because at our experimental doses, crickets were only paralyzed at that time. We also noted the physiological progression of paralysis at doses near the PD$_{50}$ values. PD$_{50}$ estimates were generated for both absolute amounts of venom injected (μg) and for weight standardized dose (μg/g cricket) using the EPA Probit Analysis Program for calculation of LC/EC values version 1.5 (SAS).

3. Results and discussion

Consistent with previous work (Binford et al., 2009), SMase D activity assays indicate striking differences between the African species and *S. rugosa* from South America (Fig. 1). Venoms from African spiders have SMase D activity similar to *L. arizonica,* whereas *S. rugosa* venom
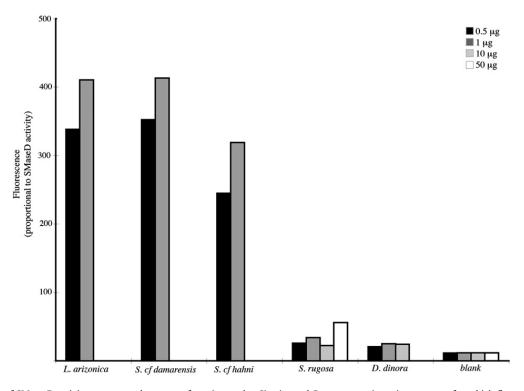
Fig. 1. Comparison of SMase D activity among crude venoms from *Loxosceles*, *Sicarius* and *Drymusa* species using an assay for which fluorescence intensity is proportional to SMase D activity. We ran reactions containing 0.5 or 1.0 μg of venom with sphingomyelin as a substrate. We assayed 10 or 50 μg venom for samples with low fluorescence activity at 0.5 or 1.0 μg based on venom availability.

is reduced to near baseline levels at concentrations that have high activity in African *Sicarius*. SMase D activity levels at low concentrations (up to 10 μg total venom protein) were comparable to those of *D. dinora* and slightly elevated above control values (Fig. 1). While the slightly elevated SMase D activity at 50 μg total venom protein for *S. rugosa* is consistent with possible sphingomyelin hydrolysis at low efficacy, this assay is prone to elevated values at high concentration of venom, even in the absence of sphingomyelin as a substrate (Binford et al., 2009). Therefore, whether or not SMase D activity is intrinsically absent from New World *Sicarius* venoms, or is highly reduced, is still under investigation. Nonetheless, it is clear that the African and New World *Sicarius* venoms differ substantially in SMase D activity (Fig. 1, Binford et al., 2009).

Both 2-dimensional SDS-PAGE of crude venom and PD_{50} estimates provide more detailed insight into patterns of variation in venom composition among *Sicarius* species than has been previously available. Separating in the second dimension expands our resolution of differences among species and reveals patterns of few proteins that co-migrate, some strikingly distinct proteins among species, and regions of proteins in the gel that overlap among species (Fig. 2). This is generally consistent with patterns of complex protein and peptide mixtures recently described in venoms of other spiders (Escoubas and Rash, 2004; Machado et al., 2005; Pretel et al., 2005; Silvestre et al., 2005; Escoubas, 2006; Escoubas et al., 2006; Kalapothakis et al., 2007).

All three *Sicarius* species have distinct patterns of crude venom proteins with very few co-migrating components

(Fig. 2B); however there are regions of the gel in which all species have high densities of proteins. For discussion, we label regions with lower-case letters (Fig. 2B a–i). Most conspicuously, stretching across the middle of the gel is a dense region of spots that likely includes expressed products of *SicTox* genes based on multiple lines of evidence. First, peptide fingerprinting of proteins in this size region of 2-dimensional separations of *Loxosceles* venoms indicate SMase D homologs are the predominant proteins in this region (Machado et al., 2005; Binford, unpublished). Secondly, the presence of multiple, paralogous forms of SMase D in diverse *Sicarius* species from Africa and South America has recently been reported by Binford et al. (2009) and estimated molecular weights of expressed proteins are between 31.2 and 32.9 kDa where there is a dense band on 1-dimensional gels (Table 1, Fig. 2A). Predicted pIs of all known *Sicarius* SMase D paralogs range from 5.1 to 9.5 (Table 1). This region likely contains other protein products such as SMase D homologs not yet captured by cDNA approaches (Binford et al., 2009), non-*SicTox* homologs, or a mixture of both (Fig. 2B a).

Outside of region a in Fig. 2B, we identify clusters of *Sicarius* venom proteins that are similar in molecular weight but migrate across a broad range of pIs. A densely staining region includes proteins slightly smaller than the *SicTox* region that spans the acidic pH range (Fig. 2B b). The abundant proteins in this region correspond with bands of the same relative size on a 1-D gel ($\sim$25 kDa), but do not resemble anything seen in 2-D gels of *Loxosceles* venoms (Machado et al., 2005; Pretel et al., 2005; Silvestre et al., 2005; Kalapothakis et al., 2007; Binford, unpublished). Based on the currently known range of characteristics of
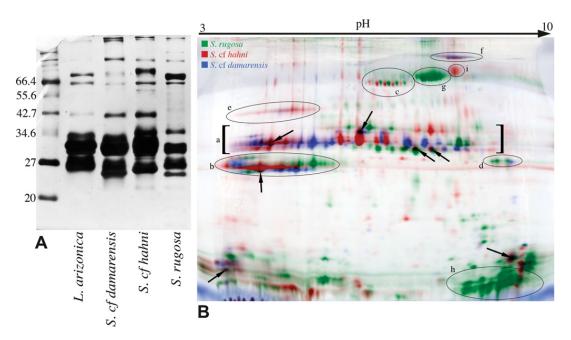
Fig. 2. Comparison of crude venoms from *Sicarius* species using 1-dimensional and 2-dimensional SDS-PAGE. A. 1-D separation of venoms from *Sicarius* species from African, South American and Central American with *Loxosceles arizonica* for comparison. The gel is 12% acrylamide with 7 μg of venom loaded in each lane and stained with silver. B. 2-D DIGE SDS-PAGE separation of crude venoms from 3 *Sicarius* species. Samples are dyed with unique fluorescent stains and run on the same gel for complete internal standardization. The gel is 15% acrylamide with a pI 3–10 gradient strip. Protein clusters discussed in the text are bracketed or circled (a–i). Arrows indicate protein spots that overlap in all 3 species.

SicTox family members, the smaller size and low pI of these proteins make them unlikely to be *SicTox* products.

A cluster of high molecular weight proteins in all three species, although less obvious in *S. cf. damarensis*, is interesting because of the repeating patterns of spots (Fig. 2B c) that is consistent with posttranslational modification, where the charged nature of the protein changes, making it more or less acidic. This finding is not surprising as many proteins spanning 20–98 kDa in *L. intermedia* (Veiga et al., 1999), *Loxosceles adelaida* and *Loxosceles gaucho* (Pretel et al., 2005) venoms have been shown to be extensively posttranslationally modified with varying forms of N-glycosylation. In fact, dermonecrotic activity has been shown to be dramatically reduced upon deglycosylation of *L. intermedia* crude venom (Veiga et al., 1999). We used the NetNGlyc program in ExPASy to predict N-glycosylation of all *Sicarius SicTox* proteins that would result from venom-expressed cDNAs and found that over 80% of the homologs we have isolated (Binford et al., 2009) are predicted to be N-glycosylated (Table 1). Predicted glycosylation sites are in the same region of the protein on all sequences which corresponds to strand 5 of the TIM barrel and is just 5′ to variable loop E of 1XX1 from *L. laeta* clone H17 (Murakami et al., 2005). Although we can make no assumptions with *Sicarius* venoms, 2-D SDS-PAGE analysis of deglycosylated *Loxosceles* venom shows an expected obvious shift in size and pI of most proteins relative to untreated venom (Binford, unpublished).

All three species have an abundance of low molecular weight components that have been evident in previous 1-D separations (*e.g.* Binford and Wells, 2003). The 1-D gel we include is a lower percentage acrylamide (12%) and did not retain small molecules (Fig. 2A). While it is likely that some low molecular weight peptides migrated off the bottom of the 2-D gel, the small molecules that were retained show substantial variation among species with only a few that appear to co-migrate. In the region captured by the gel very few migrate neutrally, indicating they are largely ionic, which is consistent with properties of neurotoxins in other spider venoms (Escoubas and Rash, 2004). Little work has been done to identify sicariid toxins that are active on insects, but three venom-

Table 1

Comparison of theoretical properties of proteins estimated from *Sicarius SicTox* cDNA sequences. We used the "compute pI/MW" tool in ExPASy (http://ca.expasy.org/) to calculate the theoretical pI and molecular weight for each known *Sicarius* sequence. To estimate these values for full length mature protein sequences we added a consensus sequence of seven amino acids obtained by 5′ RACE (ADSRRPI) for nine *Sicarius SicTox* genes (unpublished data). The values represent the range of estimates of all published sequences in the each *SicTox* clade identified in Binford et al. (2009). We used the NetNGlyc prediction program in ExPASy to predict N-glycosylation sites for each sequence.

SicTox Clade	Number of *Sicarius* sequences analyzed	GenBank accession numbers	Number of sequences predicted to be N-Glycosylated	Predicted pI	Predicted MW (kDa)
βIB	7	FJ171468–FJ171474	0	5.1–6.7	31.5–31.9
βIF	4	FJ171487–FJ171490	4	5.4–9.5	31.2–32.9
βIIA	15	FJ171491–FJ171505	15	5.5–8.5	31.7–32.6
βIIB	21	FJ171506–FJ171526	19	6.7–8.9	32.3–32.6

expressed proteins have been isolated from *L. intermedia* that have sequence homology with small peptide neurotoxins from other spiders (de Castro et al., 2004). These are roughly the size of the components in region h of Fig. 2B (5.6–7.9 kD, pI 8–9) but their specific mechanism of activity has not yet been characterized (de Castro et al., 2004).

The remaining protein migration patterns show more detailed variation across the landscape, some of which are present in only one of the species we studied. Some but not all patterns of similarity reflect degrees of relatedness (African species are more similar to one another than they are to *S. rugosa*). Consistent with phylogenetic relatedness, there are two regions of higher molecular weight proteins that span a range of pIs that are predominantly from the African *Sicarius* species (Fig. 2B e,f). One high molecular weight, and one low molecular weight cluster of proteins are unique to *S. rugosa* (Fig. 2B g,h) and are quite abundant, especially the low molecular weight components. Counter to phylogenetic relatedness, there is a band of mid-sized cationic proteins just below the *SicTox* region, that are common in *S. cf. damarensis* and *S. rugosa* but not *S. cf. hahni* (Fig. 2B d). Lastly, there is a single, moderate sized group of proteins in *S. cf. hahni*, but not the others (Fig. 2B i). With these data there is no way to discern whether these differences are due to nuances influencing protein expression or if they are fixed genetic differences. Our venom collection protocol controls for time since feeding, sex and maturity status, but small-scale variation in protein spot patterns could be influenced by differences in the number of individuals in the venom pools or other unknown factors that influence protein expression besides genetic differences between species.

Our data do not allow us to identify specific proteins, however given the relatively close relationship of *Loxosceles* and *Sicarius* (Platnick et al., 1991; Binford et al., 2008) some of the regions of proteins we detect in *Sicarius* venoms are likely to be homologs of one or more molecules known from the more extensively studied *Loxosceles*. Most detailed characterization of proteins in these venoms has focused on SMase D, but biochemical studies have also identified many other molecules. Hyaluronidase enzymes (32.5 kDa, 33 kDa, 41 kDa, 44 kDa and 63 kDa) have been identified in a number of *Loxosceles* species and are thought to act as toxin spreading factors (Wright et al., 1973; Young and Pincus, 2001; Barbaro et al., 2005; da Silveira et al., 2007). Multiple copies of metalloproteinases, serine proteases, and enzymatic inhibitors have been identified using expressed sequence tag analysis (Fernandes-Pedrosa et al., 2008), but only a few have been biochemically characterized. Two

serine proteases (85 kDa and 95 kDa) have been characterized in *L. intermedia* and shown to have gelatinolytic activity (Veiga et al., 2000). The *Loxosceles* astacin-like protease (LALP) is 30 kDa and its digestive feature has been suggested to facilitate the diffusion of other venom toxins through tissue (da Silveira et al., 2007). We are comparing sequences from cDNA libraries of *Sicarius* venom glands to infer the distribution of known toxins in this lineage.

3.1. Insecticidal activity

Consistent with differences in SMase D activity and different protein patterns from 2-D gels, we detected differences among species in insecticidal potency as estimated by PD_{50} analyses of crude venom on crickets. The PD_{50} estimates are strikingly low, ranging between 0.35 and 3.19 µg/g (Table 2), however the differences among species are significant (Table 2 ANOVA, $F_2 = 54.9$, $p > 0.0001$). We have collected as much as 480 µg of total venom protein from an individual African *Sicarius* spider (Table 2, Binford and Wells, 2003) meaning only a tiny fraction of the venom reserve of larger species is sufficient to immobilize some prey.

Intriguingly, *S. rugosa* has the lowest potency and is the representative American species that has highly reduced levels of SMase D activity (Binford et al., 2009); however, there is as much difference in potency between the two African species as there is between *S. cf. hahni* and *S. rugosa* (Table 2). It is tempting to infer that the lower potency in *S. rugosa* is influenced by the reduced or lost SMase D activity in these venoms (Fig. 1), but there are substantial differences between these species in venom components whose function is entirely unknown that likely contribute to differences in potency (Fig. 2). In fact, given the differences in protein profiles between species it is surprising to us that the PD_{50} values are as similar as they are. Moreover, the potency is still very high in *S. rugosa*. Although no work has been done on peptide neurotoxins in *Sicarius* venoms, we suspect they are present in these species based on their ubiquitousness in spider venoms, the presence of low molecular weight bands in 1-D separations (Binford and Wells, 2003), and the presence of neurotoxin homologs in *L. intermedia* (de Castro et al., 2004).

PD_{50} values are difficult to compare with published work because experimental conditions vary (*e.g.* Quistad et al., 2002; Gentz et al., 2009) and potency differs as a function of the target animal (Friedel and Nentwig, 1989; Manzoli-Palma et al., 2003; Wang et al., 2007; Herzig et al., 2008; Gentz et al., 2009). Moreover, PD_{50} values tend to be lower than LD_{50} values. At the experimental doses we used

Table 2
PD_{50} estimates of *Sicarius* venom potency. Mean venom per spider is the total venom protein in a pooled sample divided by the number of spiders from which venom was pooled (range from 2 to 7 individuals). Total body length is the length from the anterior tip of the cephalothorax to the posterior tip of the abdomen measured just before venom milking. PD_{50} values are the average and standard deviation of three replicate estimates of the dose at which 50% of crickets were paralyzed. PD_{50} µg is the absolute amount of crude venom injected and PD_{50} µg/g is the absolute amount of venom injected per gram cricket weight. Differences among species in PD_{50} µg/g are statistically significant (ANOVA, $F_2 = 54.9$, $p > 0.0001$).

Species	Mean µg venom/spider	Mean total body length (mm)	PD_{50} µg	PD_{50} µg/g
S. cf. damarensis	237.8 ± 136.8	12.0 ± 0.41	0.036 ± 0.006	0.350 ± 0.050
S. cf. hahni	200.1 ± 56.8	11.25 ± 1.5	0.146 ± 0.019	1.477 ± 0.228
S. rugosa	156.1 ± 44.4	9.4 ± 1.0	0.284 ± 0.046	3.188 ± 0.529

to capture the PD_{50} crickets were paralyzed with twitching legs and mouthparts, but not killed at the highest doses after 60 min. After 24 h in preliminary analyses, animals paralyzed at 60 min were generally dead and the other animals generally were not suggesting that paralysis is irreversible and ultimately leads to death. However, with our protocol we cannot infer LD_{50}. With the caveat that these issues constrained our comparisons, we point out some differences that may help to make our PD_{50} values more informative in comparative context with other venomous taxa. Across published estimates of paralytic or lethal doses of spider venoms on a range of target organisms (including crickets) that are standardized for venom protein per prey weight (Manzoli-Palma et al., 2003; Wang et al., 2007; Herzig et al., 2008; Gentz et al., 2009), the PD_{50} values we estimate for all three species of *Sicarius* are substantially lower than estimates of paralytic or lethal doses for all other spiders except *Latrodectus tredecimguttatus* (1.87 µg/g on roaches, *P. americanus* and 0.16 mg/kg on mice) (Wang et al., 2007) and *Loxosceles* LD_{50} on mice (0.2–4.6 µg/g) (Geren et al., 1976; Babcock et al., 1981; de Oliveira et al., 2005; Pretel et al., 2005). *Sicarius* PD_{50} estimates are lower than LD_{50} values for *L. gaucho* on crickets (20.60 µg/g), but *S. rugosa* paralytic potency on crickets is comparable to LD_{50} estimates on honeybees (3.76 µg/g) (Manzoli-Palma et al., 2003). Thus, while *S. rugosa* venoms are significantly less potent as measured by PD_{50} than venoms of their African relatives, they still are highly toxic to insects. We have much to learn about biological factors that may correlate with variation in venom potency, but we have observed no differences in diet, habitat or prey capture behavior between African and American *Sicarius* that might correlate with differences in potency on crickets. It will be informative to expand the comparison to include more prey types.

The high toxicity of *S. rugosa* venom to insects given the lack of SMase D specific activity in these venoms sets up interesting hypotheses about the functional role of SMase D activity in insect immobilization, presumably the primary role of venoms in prey capture. We can infer from our comparative PD_{50} results that, either SMase D specific activity is not in itself necessary for insecticidal activity, related but not identical activities are sufficient for insecticidal activity, or trace amounts of SMase D activity below the sensitivity of our SMase D assay are sufficient for prey immobilization. The presence of *SicTox* genes in a diverse set of American *Sicarius* that have very low, if any, SMase D activity suggests that the gene family has undergone evolution of functional specificity that has yet to be fully characterized (Binford et al., 2009). All *Sicarius SicTox* gene sequences isolated to date are in the β clade, a lineage that includes proteins that vary in levels of SMase D activity and dermonecrotic activity (Ramos-Cerrillo et al., 2004; Kalapothakis et al., 2007; da Silveira et al., 2007; de Santi Ferrara et al., 2009; summarized in Binford et al., 2009). Both African *Sicarius* species (with SMase D activity in crude venom), and American *Sicarius* species (without SMase D in crude venom) have venom-expressed β clade paralogs. We suspect that the *SicTox* homologs expressed in American *Sicarius* have evolved differences in functional specificity, and these functional variants perform a role in prey

immobilization that is analogous to that of SMase D. Preliminary data from our lab indicates that purified proteins expressed from *SicTox* genes from the α clade are highly toxic to insects. We are in the process of testing the effect of expressed *SicTox* genes from New World *Sicarius* on insects.

Conflict of interest

None.

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