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Not as docile as it looks? *Loxosceles* venom variation and loxoscelism in the Mediterranean Basin and the Canary Islands.

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Abstract

The medical importance of *Loxosceles* spiders has promoted extensive research on different aspects of their venoms. Most of the reported cases of loxoscelism have occurred in the Americas, and thus, much work has focused on North and South American *Loxosceles* species. Interestingly, loxoscelism cases are rare in the Mediterranean Basin although *L. rufescens*, endemic to the Mediterranean, is an abundant spider even in human-altered areas. Thus, it has been suggested that the venom of *L. rufescens* could be of less medical relevance than that of its congeners. In this study, we challenge this hypothesis by using multiple approaches to study venom variation in selected species and lineages from the Mediterranean Basin and the Canary Islands. We found that SMase D activity, the key bioactive component of *Loxosceles* venom, is comparable to American species that are confirmed to have medically relevant bites. The venom protein composition using SDS-PAGE presents some differences among regional *Loxosceles* taxa in banding pattern and intensity, mostly between the Canarian and *L. rufescens* lineages. Differences between these species also exist in the expression of different paralogs of the *SicTox* gene family, with the Canarian species being less diverse. In conclusion, our results do not support the challenged hypothesis, and suggest that venom of these species may indeed be as potent as other *Loxosceles* species. Pending confirmation of loxoscelism with direct evidence of *Loxosceles* bites with species identification by professionals, *Loxosceles* in the Mediterranean region should conservatively be considered medically relevant taxa.

Keywords

Spider; evolution; Sphingomyelinase D (SMase D); SDS-PAGE; *SicTox*

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Conflict of interest

The authors declare that there are no conflicts of interest.

1. Introduction

Spider venoms have become increasingly popular as a research focus because of their potential for agricultural and pharmacological applications (Chaim et al., 2011; King and Hardy, 2013; Klint et al., 2012; Windley et al., 2012). Recent conservative estimates point to the existence of more than four million molecules that selectively target a variety of vital physiological functions, providing a nearly infinite “pharmacological space” to explore (Escoubas et al., 2006). The venom of a single spider is a complex mixture of proteins, peptides and low-molecular-weight components that have evolved with the primary function to immobilize and kill prey (Chippaux et al., 1991; Zobel-Thropp et al., 2012). Although venom composition varies between closely related species, between populations of the same species and among individuals as seen in spiders (Binford, 2001; Escoubas et al., 2002; Garb and Hayashi, 2013), snakes (see Chippaux et al., 1991 for a revision), cone snails (Duda and Remigio, 2008) and scorpions (Abdel-Rahman et al., 2009), contributing factors are not well defined. Studying patterns of variation in spider venoms can provide a better understanding of mechanisms of toxin evolution, which may give clues to evaluating potential risks associated with envenomation. It can also help with developing rational antivenom strategies or fast and specific diagnoses and treatments involving medically important groups, such as the ones included in this study.

The spider family Sicariidae comprises two genera (i.e. *Loxosceles* and *Sicarius*) widely distributed in North, Central and South America, Africa and the Mediterranean Basin. Studies of crude venoms in sicariid spiders, have identified several molecules of varying sizes, some of which are proposed to be toxic (reviewed in Gremski et al., 2014) such as hyaluronidase (Young and Pincus, 2001; da Silveira et al., 2007), phospholipases-D (Kalapothakis et al., 2007), metalloproteases, serine proteases (Young and Pincus, 2001; Barbaro et al., 2005; Feitosa et al., 1998; Veiga et al., 2000) and insecticidal neurotoxins (de Castro et al., 2004). Among these components, most of the attention has focused on a family of enzymes with sphingomyelinase D (SMase D) activity. SMase D proteins belong to a multi-gene family named *SicTox* (sicariid toxin), members of which are expressed in venom glands of both *Loxosceles* and *Sicarius* (Binford et al., 2009 and references therein). Transcriptome analyses of two species – *L. laeta* (Fernandez-Pedrosa et al., 2008) and *L. intermedia* (Gremski et al., 2010) – have shown that Expressed Sequence Tags (ESTs) of SMase D represent the most abundant component of the EST expression profiling of venom glands. Sicariid venom can cause dermonecrotic lesions in humans, known as loxoscelism (*Loxosceles* reviews in da Silva et al., 2004; Vetter, 2008; *Sicarius* review in Newlands and Atkinson, 1988) but individually expressed and purified SMase D proteins injected into rabbits trigger a cascade of physiological events that lead to dermonecrosis, massive inflammatory response and lysis of red-blood cells among other effects; they have also been shown to solely reproduce the major biological effects of the whole venom (Barbaro et al., 2005; de Oliveira et al., 2005; Pretel et al., 2005; da Silveira et al., 2007; Ribeiro et al., 2007).

While much work has been done on *Loxosceles* venom from North and South American species, very little has focused on those from Africa and the Mediterranean. *L. rufescens* is widely distributed across the entire Mediterranean Basin, where it is found commonly in

human-altered habitats. This facility to cohabit with humans has expanded the distribution of the species due to human-mediated transportation and it is considered cosmopolitan (Platnick, 2014). *Loxosceles rufescens* harbors 11 different evolutionary lineages delimited using mtDNA (Planas et al., 2014) and it has been suggested that cryptic species could coexist in this area (Duncan et al., 2009). In the Canary Islands, an endemic lineage harboring several evolutionary lineages have been uncovered recently (Planas and Ribera, 2014), and in some islands, the endemic lineages coexist with *L. rufescens*, introduced by humans into the archipelago.

Although *L. rufescens* is a common synanthropic species in the Mediterranean Basin, reports on loxoscelism are scarce, but are likely more prevalent than can be inferred from the literature (Cohen et al., 1999). Published reports have been identified in the Eastern Mediterranean Basin (e.g. Israel: Cohen et al., 1999; Davidovici et al., 2006; Greece: Stefanidou et al., 2006; Turkey: Akdeniz et al., 2007; Yigit et al., 2008) while fewer come from the Western Mediterranean Basin (e.g. Spain: de Entrambasaguas et al., 2007; France: Pernet et al., 2010; Italy: Ribuffo et al., 2012). It has been widely repeated throughout literature that a bite from *L. rufescens* has far less dangerous consequences than that of its congeneric species from the Americas such as *L. laeta* or *L. reclusa*, that it is less toxic (Vetter and Swanson, 2007) or that it produces a more benign clinical picture (Cohen et al., 1999). Several lines of evidence suggest that this assumption is erroneous (Greene et al., 2009), but no study has tested this hypothesis to date. Misidentification of *Loxosceles* bites is common in the literature (Vetter and Swanson, 2007; Vetter, 2008; Gremski et al., 2014) and most of the reported cases do not follow the recommended criteria to confirm a spider bite: confirmation or observation of the bite, capture of the spider and identification by a specialized taxonomist (Isbister, 2002), complicating understanding of the actual medical relevance. News of a suspicious bite in 2006 in Gran Canaria, based on weak evidence, was echoed by local media inducing a serious public concern, a common reaction often involved in loxoscelism diagnoses (Vetter, 2008) and recently another suspicious case (R. Conca, pers. comm.) was reported from the same island.

In this study, we are interested in testing if *L. rufescens* SMase D, the key bioactive component of the venom, is different from that of other identified members of this genus that contribute to loxoscelism in humans. We investigated differences in the composition of *SicTox* genes and SMase D activity levels between *L. rufescens* and two closely related *Loxosceles* species from the Canary Islands. We also analyzed the amount of venom variation that exists within *L. rufescens*, including representatives of different lineages comprising most phylogenetic diversity in this species. Specifically, we (1) compare whole venom protein composition using SDS-PAGE, (2) compare SMase D activity levels among venoms, (3) compare the expression patterns of *SicTox* genes, and (4) analyze patterns of phylogenetic relatedness among *SicTox* genes isolated from the *L. rufescens* lineage.

2. Material and Methods

2.1 Taxon sampling

We included two endemic *Loxosceles* species from the Canary Islands: *Loxosceles* sp. GM-HI from La Gomera (GM) and *Loxosceles* sp. GC1 from Gran Canaria (GC) (Planas and

Ribera, 2014), and three representatives from both major clades of *L. rufescens* (A and B), specifically from three evolutionary lineages found in the Iberian Peninsula (A6, B2 and B3). Lineage A6 is commonly found in the Western part of the Mediterranean Basin but has been distributed worldwide due to human-mediated transportation. Lineage B2 is less common but it is also widespread across the Mediterranean, finally lineage B3 is endemic to the Iberian Peninsula (Planas et al., 2014). Detailed collecting information is available from E. Planas by request. We used *L. arizonica* for comparison in SMase D activity assays. *L. arizonica* is an appropriate reference because verified bites from this species produce similar severity of systematic effects and dermonecrosis as the better-known *L. reclusa* (Boyer et al., 2000; Boyer et al., 2010) and levels of SMase D activity of crude venoms are comparable between both species (Binford & Wells, 2003; Binford et al., 2009).

2.2 Venom extraction and quantification

We used electrical stimulation to extract venom as described in Binford and Wells (2003). Only mature females were used and venom samples were pooled among individuals to standardize for possible differences (Table 1). We pooled venom from the following number of individuals from each locality: four (A6), eight (B2), five (B3), three (GC) and six (LG). We diluted all venom in 1X Amplex Red buffer (5 mM CaCl₂, 50 mM Tris-Cl, pH 8) and quantified total venom protein using the Coomassie Plus (Bradford) Assay Reagent (ThermoScientific, Pierce).

2.3 SDS-PAGE

Crude venom proteins (5 µg) were separated from each species using one-dimensional SDS-PAGE in a 12% Criterion Precast Gel (Bio-Rad). A Broad Range Molecular Weight Standard (New England Biolabs) was loaded for size reference. Proteins were visualized using a standard silver stain protocol.

2.4 SMase D Activity Assay

We assayed SMase D activity using a modification of the Amplex Red Phospholipase D Assay Kit (Molecular Probes, Invitrogen) as described in Binford et al. (2009) with sphingomyelin as the substrate. Fluorescence emission was measured from reactions that contained 0.5 µg of crude venom and each reaction was conducted twice. We used *L. arizonica* venom as a positive control for activity comparisons. For negative controls we substituted 1x Amplex Red Buffer for venom and ran the reactions with and without sphingomyelin, and also conducted one reaction for each species without sphingomyelin. We used a Perkin Elmer LS55 Luminescence Spectrometer (Waltham) for all measurements where an increase of fluorescence emission directly correlates to SMase D activity.

2.5 SMase D Expression Patterns

2.5.1 cDNA synthesis, PCR amplification and cloning—Two days after venom extraction, we dissected and removed venom glands and immediately froze them in liquid nitrogen (Table 1). For RNA isolation and cDNA synthesis, we followed the same procedure as described in Binford et al. (2009) using the ChargeSwitch® Total RNA Cell kit (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen). SMase D was

amplified using a pair of primers (“sphing1f” 5’ - TGGATHATGGGNCAAYATGGT- 3’ and “anch2” 5’ - CCACGCGTCGACTAGTAC- 3’) that were previously used to successfully amplify a diverse set of SMase D homologs from a wide range of *Loxosceles* and *Sicarius* species (Binford et al., 2009). These primers amplify a fragment of ~1000 bp, including most of the mature protein and the poly-A tail. For PCR amplification, we used 0.2 µl of each cDNA pool, 5 µl MasterAmp F PreMix (Epicentre Technologies, 1x final concentration), and recombinant Taq Polymerase (New England Biolabs, 0.05 U/µl) in a total volume of 10 µl. The annealing temperature for all reactions was 60° C.

We cloned PCR products (~1 kb) into pCR®4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen). We screened bacterial transformants using PCR under the same optimal conditions used to amplify the inserts. Colonies with positive products were grown in selective medium and purified using the QIAprep spin mini-prep kit (Qiagen). Inserts were sequenced for both directions with T3 and T7 primers at the Genomic Analysis and Technology Core (University of Arizona).

2.5.2 Sequence analysis and phylogenetics—Contigs were assembled and edited in Geneious 4.6.5 (Drummond et al., 2009). We searched all sequences using tBLASTx against the NCBI nucleotide database (<http://blast.ncbi.nlm.nih.gov/>) to confirm SMase D homology. We aligned all sequences using Clustal X (Thompson et al., 1994) and translated them into amino acid sequences using Geneious. Only coding regions were used for further analysis and we removed from the data set sequences that had early stop codons or frame-shift indels.

To investigate phylogenetic relationships, we used a subset of our data that included sequences that were minimally 5% different in amino acid sequence divergence for each species or lineage. We aligned these sequences with those from Binford et al. (2009) that follow the same criteria, using the published alignment as a guide. We analyzed the data set in a Bayesian framework using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003). We conducted two simultaneous runs, each with four Markov Chain Monte Carlo chains for 2×10^6 generations, sampling every 100 generations. We confirmed convergence by examining the standard deviation of the split frequencies between the two simultaneous runs and the Potential Scale Reduction Factor (PSRF) diagnostic. We performed the burn-in discarding the first 5000 trees of each run and we constructed a majority-rule consensus tree from the remaining trees.

We tested for significant difference in the set of expressed SMase D paralogs using a chi-squared test ($p < 0.05$). The significance value of the chi-squared test was obtained with a Monte-Carlo simulation using 10,000 replicates due to the low number of paralogs expressed in some clades. Statistical analyses were conducted in R (R Core Team, 2014).

3. Results and Discussion

3.1 Crude Venom Analysis

SDS-PAGE analysis of crude venom extracted from individuals belonging to different *L. rufescens* lineages (A6, B2, B3) and novel Canarian species (LG, GC) are shown in Figure

1. Protein clusters corresponding to the molecular weights of known *Loxosceles* venom components are evident with differences in composition, number and intensity of bands across all species (Fig. 1). Tentative identification of known components likely included in bands was based on molecular weight, although further proteomics analyses are needed to corroborate their identity with confidence.

Comparing venoms based on one-dimensional gel migration of proteins is challenged by issues such as differential loading of venoms resulting in slight differences in total protein abundance and visibility of specific bands, and bands being heterogenous mixtures of proteins and peptides. Nonetheless, banding patterns tend to be repeatable within sexes from individuals in the same population or species (Binford, pers. obs.), making them a good heuristic comparison. The patterns of protein migration across taxa included here are comparable to *L. rufescens* individuals collected in North America (Binford et al., 2009). The degree of difference is more acute between *L. rufescens* and the two Canarian *Loxosceles* species, but some differences also exist within *L. rufescens* lineages. In high molecular weight regions, differences exist between A6 and the other two *L. rufescens* lineages (B2 and B3), where the former lack many bands that are conspicuous in B2 and B3 (Fig. 1Ai). Differences in this band range are also clear between *L. rufescens* and the Canarian species. A remarkable difference is observed in a dense band of ~63 kDa that is present only in LG (Fig. 1Aii). The most conspicuous bands in all cases were those situated between 30 and 36 kDa (Fig. 1B), that correspond to the estimated molecular weight of expressed proteins of *SicTox* gene family (Binford and Wells, 2003; Binford et al., 2009 and references therein). The density of the band was higher in B2 and less dense in GC. GC also lacks the 35 kDa band found in the rest of the representatives. There are further differences in banding patterns of proteins ranging from 21 to 27 kDa (Fig. 1C). Here, all three *L. rufescens* lineages show a conspicuous band in the ~27 kDa which is not visible in the Canarian species. The two Canarian species also lack the tenuous ~21 kDa band found in all three *L. rufescens* lineages. Although we can only speculate about the identity of these bands, their sizes match well with the 23 kDa fibrinogeneolytic protease and the 27.5 kDa gelatinolytic protease identified by Young and Pincus (2001), both belonging to the *Loxosceles* astacin-like proteases (LALP). These proteins have been suggested to play an important role in the spreading of other noxious components of the venom by degrading extracellular matrix molecules and rendering tissues more permeable (da Silveira et al., 2007; Trevisan-Silva et al., 2010; Gremski et al. 2014). A varying pattern of low molecular weight bands (Fig. 1D) is found in all the *L. rufescens* lineages, GC is remarkably different spanning size and intensity of this region. These bands most likely correspond to venom peptides (3 kDa - 10 kDa) known to be insecticidal, neurotoxic and abundant in most spider venoms but poorly understood in *Loxosceles* (Chaim et al., 2011). While some differences in detectability of relatively faint bands could be influenced by subtle differences in total protein loaded, we are convinced that the differences in presence/absence and relative size are sufficient to be real.

3.2 SMase D Activity

All species tested show high levels of SMase D activity in crude venom with no significant differences among them, or from the positive control (i.e. *L. arizonica*) (Fig. 2). The amount

of fluorescence emitted in assays from all *L. rufescens* lineages is consistent with *L. rufescens* results reported by Binford et al. (2009) and equivalent to the Canarian species. If the venom from *L. rufescens* is less potent than other *Loxosceles* species as the result of reduced SMase D activity, then we would expect to see lower amount(s) of SMase D activity, but we do not. This high enzyme activity, in conjunction with intense SMase D bands in the 30–35 kDa region on the protein gel in Figure 1 suggests that *L. rufescens*, and the two Canarian species, contain *SicTox* proteins and SMase D activity and have the potential to be as potent as other *Loxosceles* species when comparing the same amount and concentration of venom. Further studies, especially bioassays, should be conducted to confirm this hypothesis.

3.3 SMase D phylogenetic diversity and expression patterns

We isolated a total of 325 SMase D homolog sequences. Of these, 301 were non-redundant at the nucleotide level and 262 were nonredundant at the amino acid level (Table 1). The phylogenetic analysis performed with a selection of sequences that were 5% divergent at the amino acid level (35) obtained in this study (GenBank accession numbers [KM363195](#) to [KM363228](#)) together with those of the alignment used in Binford et al. (2009), showed an almost identical topology compared to the aforementioned study (Fig. 3). In most of the lineages, the topology obtained within individual *SicTox* paralogs mirrored the phylogenetic relationships between *L. rufescens* and the Canarian species (Planas and Ribera, 2014).

The SMase D paralogs that were most abundant in all studied species belong to clade α IC (Fig. 4). This clade was reported to exclusively include members of the closely related *gaucho*, *amazonica* and *rufescens* species groups (Binford et al., 2009). Here we also include two species endemic to the Canary Islands (Planas and Ribera, 2014). In both Canarian species, more than 90% of the sequences belong to this clade, while in the *L. rufescens* lineages, this clade represents a maximum of 79% in B3 and a minimum of 71% in A6. Within this clade, paralogs from the two main subclades were found: α IC1 and α IC2. In all cases, the representatives of clade α IC1 were more common than the α IC2 clade. In LG and GC, α IC2 was roughly seven and four times less represented than α IC1, respectively. The difference was also high in B2 and B3, where representatives of α IC2 clade were 15 and five times less represented than α IC1 clade members, respectively. In A6, this difference was less marked, though α IC1 clade was only two times more represented than α IC2 clade. To date, only *L. rufescens* sequences reported in Binford et al. (2009) were included in the α IC2 clade; here, we have expanded this representation by adding members of the aforementioned Canarian species.

Sequences from species in this study are also represented in the α II *SicTox* clade; the Canarian species were less numerous than in the *L. rufescens* lineages. B2 was the only lineage that has one sequence belonging to α II2 clade, while all the other sequences belong to α II1 clade. Until now, the only α II2 clade sequence is from *Loxosceles intermedia* (LoxTox i5 in Kalapothakis et al., 2007) while α II1 clade is formed by representatives of *gaucho*, *amazonica* and *rufescens* species groups, and by North American representatives of *spadicea* and *reclusa* groups. The last *SicTox* clade represented in our analysis is the α IV1 clade. In this case, no Canarian sequence is represented and only sequences from the *L.*

rufescens lineages were included. In this study, we recovered *SicTox* α clade representatives, but not any from the β clade. There is, however, evidence that β clade homologs are present in *L. rufescens* venom from an introduced North American population that belongs to lineage A6, using MudPIT proteomic analysis (Binford et al., unpublished). In addition, cDNA library sequence analysis of A6 lineage has identified several *SicTox* homologs from both α and β clades representing 7% of the whole venom gland transcriptome (Binford et al., unpublished).

We found significant differences among the SMase D expression patterns (i.e. different proportion of expressed paralogs) ($p < 1e^{-05}$). These differences are also significant when we compare the expression patterns among the three members of *L. rufescens* ($p = 0.0018$). Conversely, we haven't found differences between the two Canarian species ($p = 0.4456$) nor between A6 and B3 ($p = 0.3626$). Sequences were ascribed to known *SicTox* clades following the nomenclature proposed by Binford et al. (2009).

3.4 Patterns of venom variation

The Canarian species studied seem to show less complexity in venom than the three *L. rufescens* Mediterranean lineages. We found this pattern in the SDS-PAGE analysis of crude venom and also in the SMase D expression analysis, where evidence of a lack of expression of some paralogs of the *SicTox* gene family in the Canarian species was found (Fig. 1). Although we could not discard the possibility that we failed to sequence those paralogs, it seems rather improbable because we sequenced more clones from transcript pools of the two representatives of LG and GC, than from the *L. rufescens* lineages (Table 1).

It has been widely observed that when snakes and *Conus* snails change their diet, and thus prey preferences, changes in venom composition may also occur (Daltry et al., 1996; Duda, 2008; Duda and Lee, 2009). In spiders, there is less evidence of differences in venom composition related to diet preferences. However, work by Binford (2001) demonstrated a reduction on the relative proportion of low molecular weight components that was evolutionarily coincident with a shift from orb-weaving to wandering and an increase in diet breadth. Also, a recent study on myrmecophagous spiders of the genus *Zodarion*, (Pekár et al., 2012) reported that venom composition was markedly different and correlated with prey preference. In the same study, they reported that in a *Zodarion* specific biotype from the Macaronesian island of Madeira, the fact that different ant species inhabit the island has promoted changes in body size and probably in venom. Although specific studies in diet preferences of *Loxosceles* species are few (Zobel-Thropp et al., 2012), field and laboratory observations suggest that they are generalist predators. Since the venom properties should be heavily dependent on the differences in diet and behaviors associated with the capture and digestion of the prey, the simplification of venom in the Canarian species could be due to a less diverse prey range found in the islands where they are distributed.

3.5 Loxoscelism in the Mediterranean basin

In the Mediterranean basin, well-reported cases of loxoscelism are scarce and more efforts should be conducted to provide reliable identification of spiders involved in bites or to determine the presence of *Loxosceles* venom at the bite site (Akdeniz et al., 2007). Among

the published cases, the ones reported from the Eastern Mediterranean seem more abundant and acute than in the Western regions. Although this could be due to a bias in the medical prevalence of reporting these cases, it could also be explained by differences in the dermonecrotic consequences of the *Loxosceles* bite. Lineages A6 and B2 are distributed across the Mediterranean (Planas et al., 2014) and we demonstrate evidence of differences in venom, especially in SMase D expression patterns. In order to relate these differences to a differential dermonecrotic activity, further studies need to assay the specific effects on vertebrates of every SMase D clade found in the venom of *L. rufescens* (i.e. α IC, α II and α IV), as they have never been studied in such detail. Although some caution should be taken when relating SMase D activity to loxoscelism (Lopes et al., 2013), the dermonecrotic effects of a wide phylogenetic range of *Loxosceles* venom is firmly established. Also, the broad and consistent expression of multiple SMase D paralogs found in all the species is reasonable evidence of potential for bites of these species to cause loxoscelism. Thus, the venom of *L. rufescens* and close relatives should be conservatively considered to be potentially as active as other *Loxosceles* with known medically relevant bites. Pending a definitive assessment of the toxicity of *L. rufescens* and related species, caution should be taken with *Loxosceles* bites in the Mediterranean Basin, the Canary Islands, and the expanded range of *L. rufescens*.

To gain reliable reports for further studies of loxoscelism in these areas, the research community would benefit from more clear communication with medical personnel about our need for better data. Unfortunately, in neither of the two cases of loxoscelism that have been reported from the Canarian Islands were the necessary steps followed to unambiguously relate them to a spider bite. The situation is also complicated by the likely reality that, as in better-studied *Loxosceles* lineages, many, if not most, cases of envenomation may imply no or minor reactions in humans (Vetter and Isbister, 2008). Envenomation by *Loxosceles* spiders most commonly takes place accidentally and in urban habitats. In the Canary Islands, *L. rufescens* has a tendency to live in human-altered areas while the endemic species are found in less disturbed areas (Planas and Ribera, 2014). Thus, if there are loxoscelism cases in the Canary Islands archipelago, the introduced *L. rufescens* is more likely to be the culprit than the endemic species. With improved understanding of the actual risks associated with bites of spiders in the *L. rufescens* species group, we will be better able to educate the public about actual risks associated with *Loxosceles*, and be better prepared to diagnose and treat loxoscelism in the region.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Venom SMase D in *L. rufescens* species group is comparable to American species
- Multiple *SicTox* paralogs are venom-expressed in the *L. rufescens* species group
- Notable differences between *L. rufescens* and two Canarian species venoms
- Evidence of subtle differences in venoms among *L. rufescens* lineages
- Venom of these species may pose comparable medical risks as American *Loxosceles*

Figure 1_.

SDS-PAGE comparison of crude venoms from three lineages (A6, B2 and B5) of *Loxosceles rufescens* and two *Loxosceles* from the Canary Islands (GC and GM). Main banding clusters are labeled (A–D) for reference in the text. Schematic tree representing the phylogenetic relationship between *L. rufescens* lineages (A6, B2 and B5) and Canarian species. Broad range molecular weight markers are labeled for size reference.

Figure 2 _.

SMase D-specific activity of 0.5 mg of total protein from crude venom from each *Loxosceles rufescens* lineage (A6, B2 and B3) and from the two Canary Islands *Loxosceles* species (LG and GC). We used *L. arizonica* for positive control and substituted 1 x Amplex Red Buffer for venom in blank.

Figure 3 _.

Phylogenetic tree obtained from Bayesian inference analysis of the combined dataset of SMase D sequences that were minimally 5% different in amino acid sequence divergence for each species or lineage (colored accordingly) obtained in this study and those from Binford et al. (2009) that follow the same criteria. Black dots in nodes indicate posterior probability support > 0.95.

Figure 4 _.
Relative proportions of SMase D paralogs expressed in each *Loxosceles rufescens* lineage (A6, B2 and B3) and from the two Canary Islands *Loxosceles* species (LG and GC). n = number of sequences.

Table 1 _

Number of individuals used for SMase D expression analysis and total number of expressed SMase D homologs, and nonredundant sequences at nucleotide and aminoacid level.

Species	Individuals in mRNA pool	SMase D homologs recovered	Nonredundant nucleotide sequences	Nonredundant aminoacid sequences
<i>L. rufescens</i> A6	3	63	60	53
<i>L. rufescens</i> B3	2	40	38	37
<i>L. rufescens</i> B2	4	62	54	51
<i>Loxosceles</i> sp. GC	3	72	65	55
<i>Loxosceles</i> sp. LG	4	88	84	66
Total	16	325	301	262