Article

Fossilized Venom: The Unusually Conserved Venom Profiles of *Heloderma* Species (Beaded Lizards and Gila Monsters)

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Abstract: Research into snake venoms has revealed extensive variation at all taxonomic levels. Lizard venoms, however, have received scant research attention in general, and no studies of intraclade variation in lizard venom composition have been attempted to date. Despite their iconic status and proven usefulness in drug design and discovery, highly venomous helodermatid lizards (gila monsters and beaded lizards) have remained neglected by toxinological research. Proteomic comparisons of venoms of three helodermatid lizards in this study has unravelled an unusual similarity in venom-composition, despite the long evolutionary time (~30 million years) separating *H. suspectum* from the other two species included in this study (*H. exasperatum* and *H. horridum*). Moreover, several genes encoding the major helodermatid toxins appeared to be extremely well-conserved under the influence of negative selection (but with these results regarded as preliminary due to the scarcity of available sequences). While the feeding ecologies of all species of helodermatid lizard are

broadly similar, there are significant morphological differences between species, which impact upon relative niche occupation.

Keywords: adaptive evolution; venom; toxin; Heloderma; beaded lizard; gila monster

1. Introduction

There are five extant species of helodermatid lizards: *Heloderma alvarezi*, *H. charlesbogerti*, *H. exasperatum*, *H. horridum and H. suspectum* [1,2]. *H. suspectum* last shared a common ancestor with the other extant species approximately 30 million years ago. *H. exasperatum* and *H. horridum*, the other two species included in this study, last shared a common ancestor approximately 4 million years ago. All are native to the south-western part of the North American continent and inhabit rocky, semiarid and scrubland habitats. Such an absence of appreciable intraclade diversity in ecology is reflected in the overall extreme similarity in morphology of the species. These lizards are also characterised by having a very low metabolic rate and are known to exhibit a preference for low-body temperatures, spending most of the year at temperatures lower than 25 °C [3]. *Helodermatid* lizards raid the nests of birds and rodents [4] but may also predate upon adult rodents [5].

The genus *Heloderma* has been recognised as venomous for more than a century. The teeth are deeply grooved and the glands are very large. Most cases of human envenomation involve lizards biting and holding with their strong jaws, sometimes for hours. Envenomations by helodermatid lizards may be clinically complex, with symptoms including extreme pain, acute local swelling, nausea, fever, faintness, myocardial infarction, tachycardia, hypotension, and inhibition of blood coagulation [6–11]. Studies of helodermatid lizard venom have identified several components (Table 1). Of these, exendin-4, isolated from *Heloderma suspectum* venom, is a peptide agonist of the glucagon-like peptide (GLP) receptor that promotes insulin secretion. It has been clinically used to treat type 2 diabetes and to enhance plasma insulin secretion [12].

Variation in venom profiles has been extensively documented between snake species of the same genus [13–25] and between individuals of the same species, with intraspecific differences found among different geographic localities [16,26–31], and between juveniles and adults [28,32–34]. Such taxonomic, geographic and ontogenetic variation has been linked to strong natural selection in response to differing prey species [17,28,31,35–41].

Traditionally, reptile venom research has focused mostly on clinically important snake species. As a result, our understanding of the evolution of helodermatid lizard venom is limited. In this study, we compare the venom proteomes of *H. exasperatum*, *H. horridum* and *H. suspectum* in order to gauge the extent of the diversification in venom composition that has occurred over 30 million years since these species last shared a common ancestor [1].

Table 1. *Heloderma* venom peptides/proteins which have been proteomically characterized.

Protein Type/ Toxin Class	Toxic Action	Uniprot Accession #(s)
CRiSP (cysteine rich secretory protein)	Paralysis of peripheral smooth muscle and induction of hypothermia through blockage of various channels including ryanodine and L-type calcium channels.	Q91055
Exendin	Induces hypotension via relaxation of cardiac smooth muscle.	C6EVG1, C6EVG2, P04203, P04204, P20394, P26349
Helofensin	Lethal toxin that inhibits direct electrical stimulation of the isolated hemi-diaphragm.	C6EVG6, D2X5W3, D2X5W4, Q7LZ31
Kallikrein	Increase of vascular permeability, production of hypotension, stimulation of inflammation in addition to cleavage of fibrinogen.	P43685, C6EVG4, C6EVG5
B-type Natriuretic peptide/helokinestatin precursor	Natriuretic peptides produce hypotension through the relaxation of aortic smooth muscle. The helokinestatin peptides are antagonists of bradykinin at the B2 bradykinin receptor.	C6EVG7, D7FB56, D7FB57, E8ZCG5
Phospholipase A_2 (Type III)	Inhibition of platelet aggregation via the epinephrine-induced pathway.	C6EVG9, C6EVH0

2. Results and Discussion

Shotgun sequencing recovered toxin types previously known from the Heloderma venom proteome: CRiSP, exendin, kallikrein, helokinestatin and Type III phospholipase A_2 (Table S1). In addition, this analytical technique recovered types previously known only from transcriptome studies: hyaluronidase, natriuretic peptide and nerve growth factor.

One-dimensional gel electrophoresis (1D-GE) utilizing the tris-tricine method indicated a gross overall similarity between the three venoms (Figure 1). For each species there was a notable difference between non-reduced (NR) and reduced (R) samples. Most notably, a 100 kDa band was present in the non-reduced lanes but absent from the reduced lanes. Conversely, the reduced lanes exhibited a dark band at 50 kDa not present in the non-reduced, indicating that the 100 kDa band was a disulfide-linked dimer.

2D gels (2D-GE) confirmed the strikingly similar nature compositions of the three venoms (Figures 2–4, Table S2). While the venoms are broadly similar in overall protein composition, it is clear that there are significant differences in relative expression levels. This was most apparent in the PLA_2 region. While these variations may point towards differential evolution, they may be also the result of intergel variation or simply arbitrary variation in venom gland content between individuals at the time of milking. More extensive comparative sampling is required to elucidate individual variation *vs.* species level variations. Regardless, the overall protein composition was vastly more conserved than has been noted even for closely related species of snake (*cf.* [42]).

Figure 1. Reduced and non-reduced tris-tricine 1D-gel comparison of *H. exasperatum*, *H. horridum* and *H. suspectum*.
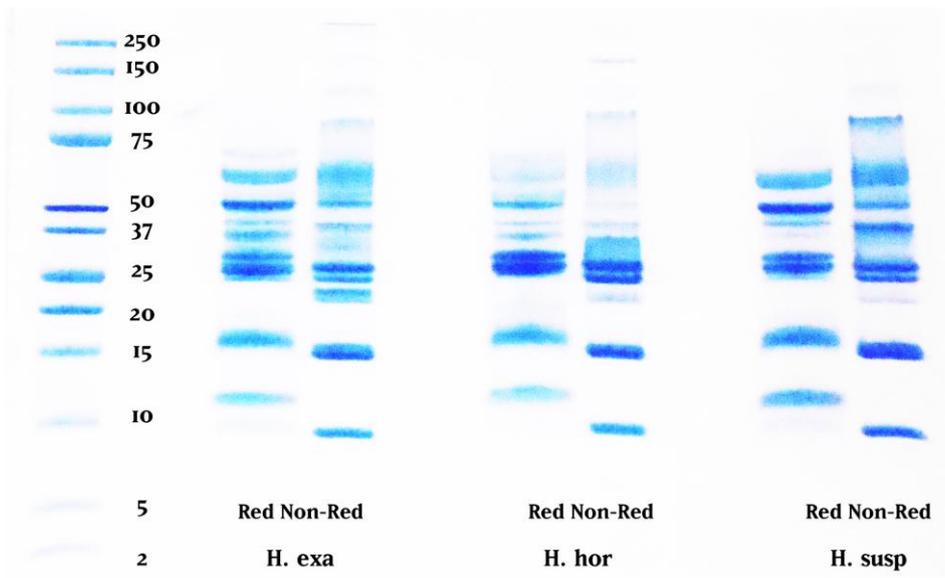

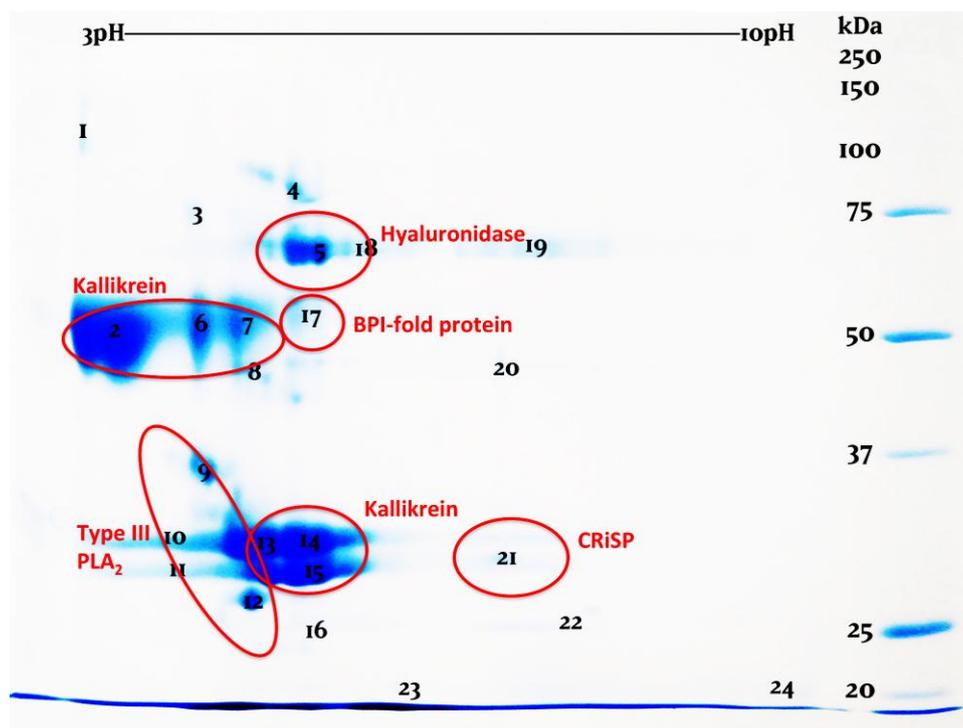**Figure 2.** 2D-gel examination of *H. exasperatum* venom.

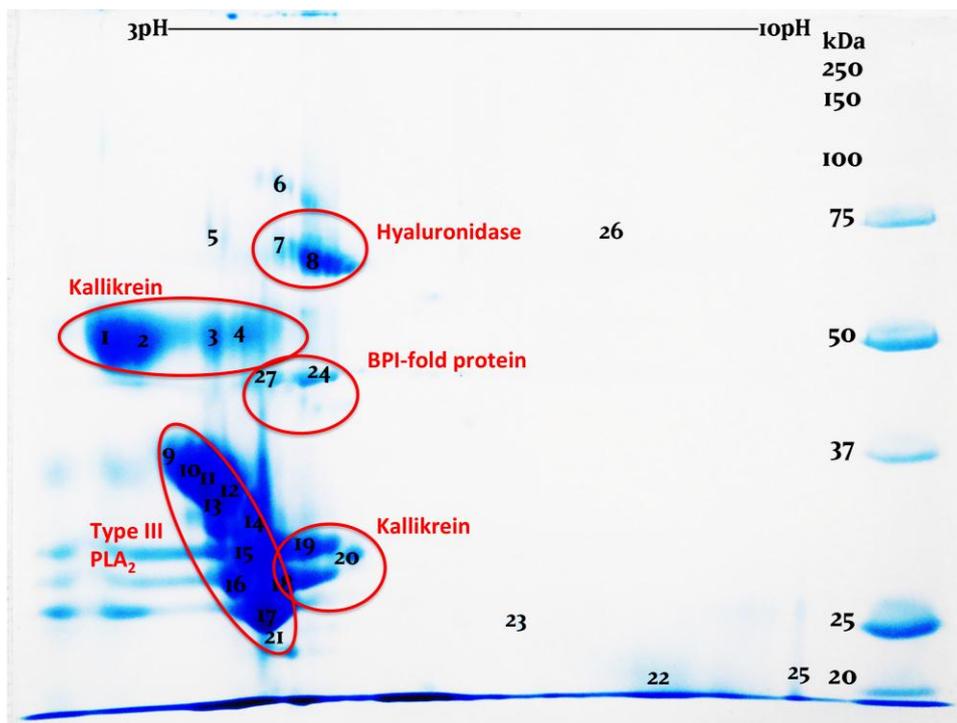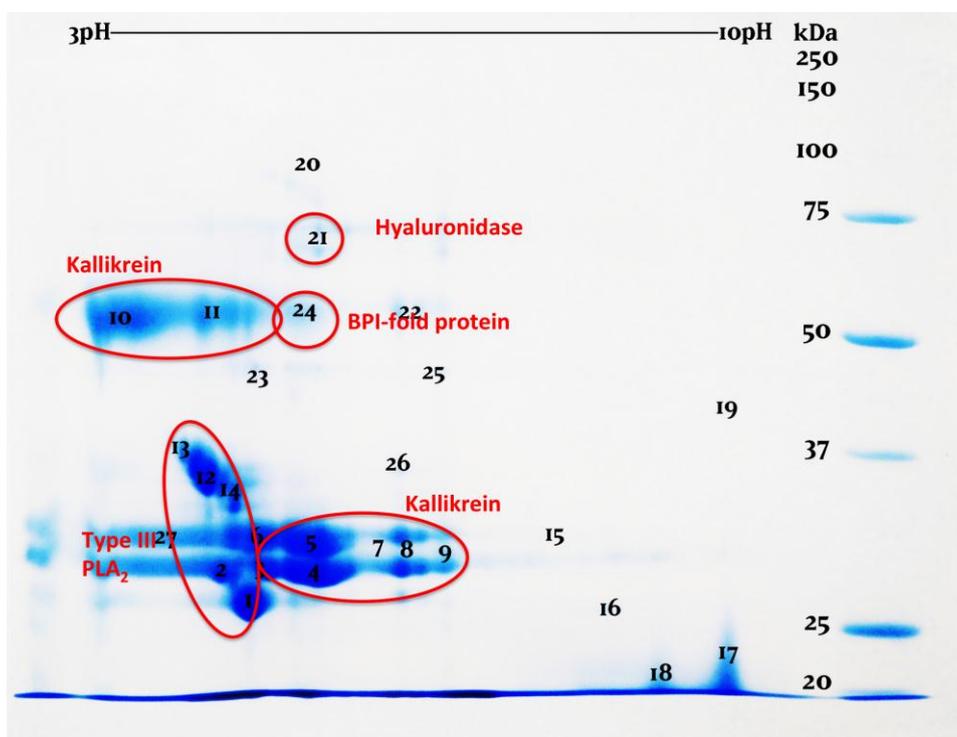**Figure 3.** 2D-gel examination of *H. horridum* venom.

Figure 4. 2D-gel examination of *H. suspectum* venom.

2D-GE revealed in all venoms previously unknown venom components which were identified as bactericidal/permeability-increasing (BPI)-fold (all species) and semaphorin proteins by searching the LC-MS/MS results against our previously constructed *H. suspectum* venom gland cDNA library [43,44]. While semaphorin was identified in *H. suspectum* only, it must be noted that light spots located in similar regions of the *H. exasperatum* and *H. horridum* gels, for which mass spectrometry

analysis was unable to provide an identity, suggest that this component is likely present in the other *Heloderma* venoms. mRNA sequences are given in Supplemental File 1 and genbank accession numbers are KP224275 (BPI-fold) and KP224276 (semaphorin). Phylogenetic analysis for both protein types identified *Anolis* genome sequences as the nearest known relative (Figures 5 and 6). The fact that both of these were identified as being transcribed by the venom gland indicates that they are indeed secreted by this gland and are not mucus contaminants. Therefore, they may play an as yet unidentified role in envenomation.

The precise role of venom in the ecology of helodermatid lizards remains unknown. Beck [45] considered it a "paradox" that helodermatid lizards hold on "with bulldog tenacity" when biting in apparent defense, thus increasing the lizard's chance of injury or death. However, this assertion merely reflects a common fallacy of evolutionary thinking—that the individual lizard is the "unit of selection". As selection takes place at the level of the gene, the death of the individual lizard does not preclude strong selection for "bulldog tenacity" in defensive bites, as presumably this tactic maximises the unpleasantness of the encounter for the aggressor, thus ensuring it avoids such encounters in future. However, the overall pharmacological profile of the venom includes components with lethal neurological effects or other severe physiological targeting, actions not consistent with a purely defensive role for the venom as defensive toxins are typically pain inducing [36]. Rather such lethal effects point towards the venoms having at least some role in predation.

Figure 5. Phylogenetic reconstruction of (**A**) BPI-fold and (**B**) semaphorin proteins. Previously known sequences are referred to by their uniprot accession codes while *Heloderma suspectum* sequences obtained in this study are referred to by their genbank codes.
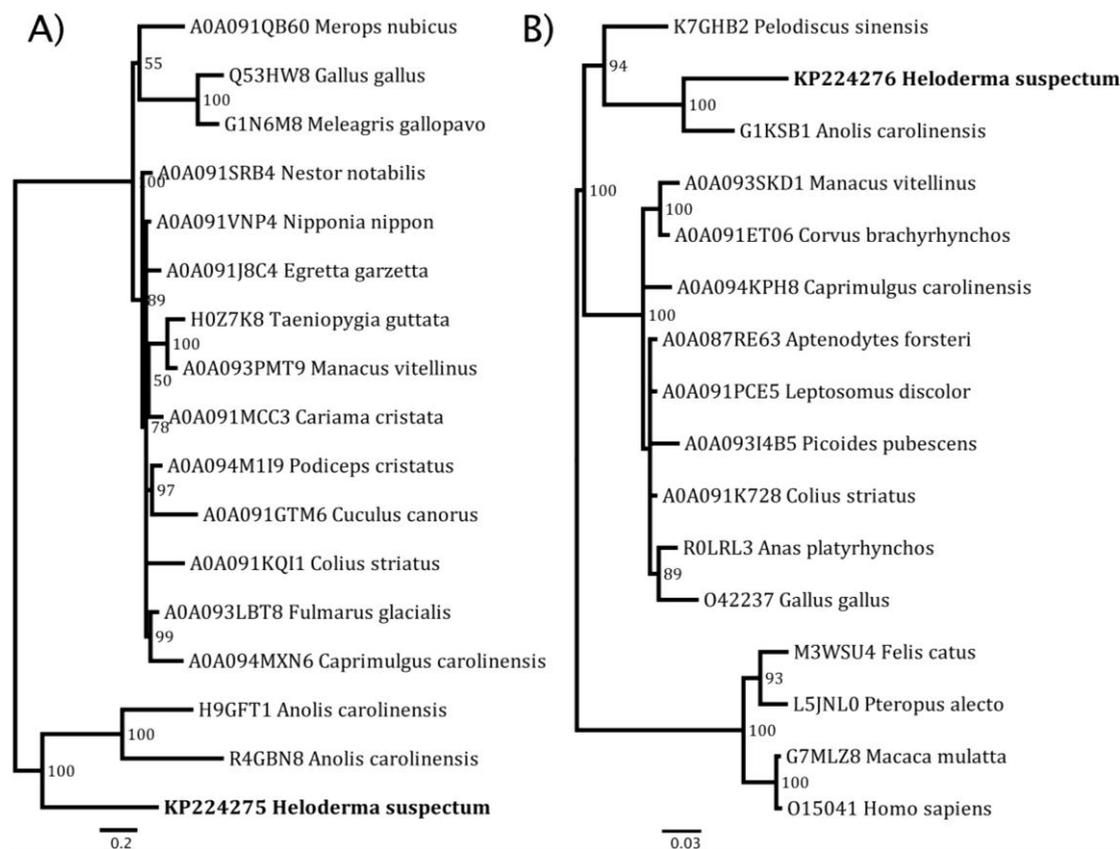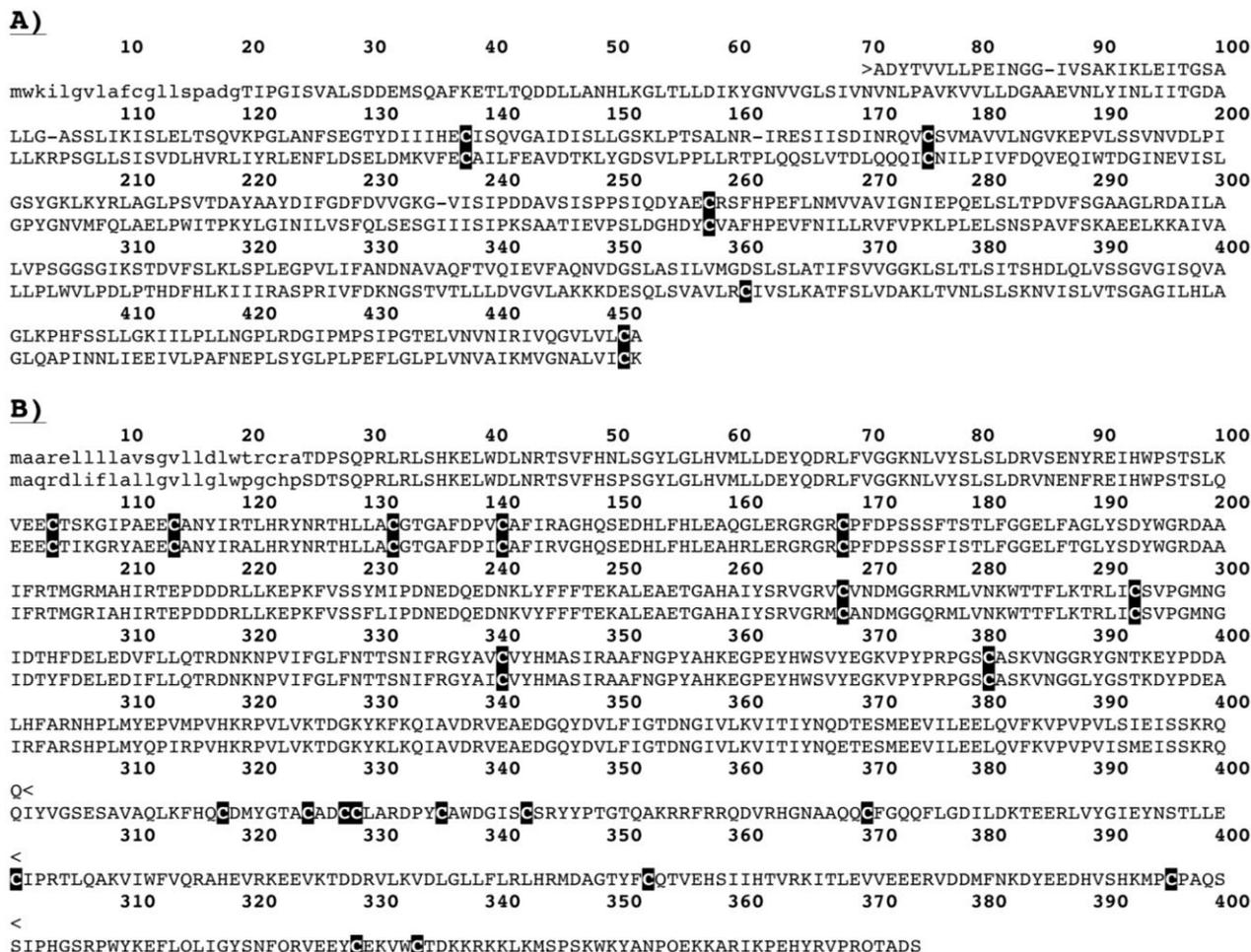
Figure 6. Sequence alignment of (**A**) the BPI-fold proteins from *Heloderma suspectum* venom (KP224275) and *Anolis caronlinensis* genome (R4GBN8) and (**B**) the semaphorin proteins from *Heloderma suspectum* venom (KP224276) and *Anolis caronlinensis* genome (G1KSB1).

Very few sequences are available and most are from *H. suspectum* and thus selection analyses must therefore be regarded as extremely preliminary. Regardless, pairwise-estimation of omega (non-synonymous (dN) to synonymous (dS) ratio) in this study revealed a greater influence of negative selection on the major toxin-encoding genes of *Heloderma* lineage (Table 2), even for comparisons between *H. horridum* and *H. suspectum* sequences. The absence of diversity in venom-composition and toxin-encoding genes within the sister *Heloderma* lineages suggest that the venoms are not evolving under the diversifying selection pressure characteristic of the predator-prey "chemical arms races" in which venomous organisms utilising their venom for prey subjugation find themselves [31,35,36,41]. However, as noted above, there are obvious variations in relative expression levels within toxin classes and thus these relative expression levels may be a novel form of diversification.

While the venom has actions consistent with predatory use, a defensive venom role is also supported by the aposematic colouration of helodermatid lizards, the fact that these lizards are slow moving and vulnerable above ground (the osteoderms in their skin are another line of defense against would-be predators), and the fact that the lizards often feed on "defenseless" prey such as eggs and nestlings [3].

That being said, the results of the present study do not refute the hypothesis that helodermatid lizard venom may be used (at least partially) for predation, as all species occupy similar ecological niches and therefore feed on similar prey items. It must be emphasised that some toxins have actions consistent with predatory effects including lethal effects upon blood pressure, coagulation and neurological function. More in-depth analyses of the venom gland transcriptomes of helodermatid lizards to mine "enough" nucleotide sequences for evolutionary selection analyses may shed light in this regard.

Table 2. Relative toxin molecular evolutionary rates.

Toxin Type	Sequence pairs	Estimates
Kallikrein	EU790962.1 (*H. suspectum*) *vs.* HM437246.1 (*H. horridum*)	dN: 0.180; dS: 0.225; dN/dS: 0.80
	EU790963.1 (*H. suspectum*) *vs.* HM437246.1 (*H. horridum*)	dN: 0.242; dS: 0.450; dN/dS: 0.53
	EU790962.1 (*H. suspectum*) *vs.* EU790963.1 (*H. suspectum*)	dN: 0.081; dS: 0.173; dN/dS: 0.47
	Average	dN: 0.167; dS: 0.282; dN/dS: 0.60
CRiSP	EU790958.1 (*H. suspectum*) *vs.* U13619.1 (*H. horridum*)	dN: 0.011; dS: 0.022; dN/dS: 0.49
Helofensin	GQ918270.1 (*H. suspectum*) *vs.* EU790964.1 (*H. suspectum*)	dN: 0.030; dS: 0.036; dN/dS: 0.84
	GQ918271.1 (*H. suspectum*) *vs.* EU790964.1 (*H. suspectum*)	dN: 0.052; dS: 0.065; dN/dS: 0.80
	GQ918271.1 (*H. suspectum*) *vs.* GQ918270.1 (*H. suspectum*)	dN: 0.020; dS: 0.027; dN/dS: 0.74
	Average	dN: 0.034; dS: 0.042; dN/dS: 0.80

3. Experimental Section

3.1. Venom Collection

Venoms were obtained from captive bred adult male specimens of *Heloderma exasperatum* (Rio Fuerte, Mexico founder stock), *Heloderma horridum* (Colima, Mexico founder stock) and *Heloderma suspectum* (Phoenix, Arizona, USA founder stock).

3.2. Shotgun Sequencing

In order to identify low molecular weight peptides that do not resolve well on 1D or 2D gels, shotgun sequencing was used. Three μg of crude venom sample was dissolved in 50 μL of 100 mM ammonium carbonate to reduce and alkylate cysteine bonds with subsequent addition of 50 μL of 2% iodoethanol/0.5% triethylphosphine in acetonitrile. The sample was afterwards resuspended in 20 μL of 40 mM ammonium bicarbonate, before overnight incubation (at 37 °C) with 750 ng of sequencing grade trypsin (Sigma-Aldrich, Castle Hill, Australia). To stop digestion, 1 μL of concentrated formic acid was added to each of the samples. Samples were lyophilised then resuspended in 20 μL of 5% ACN/0.5% FA, put into MS vials and subjected to LC-MS/MS analysis.

3.3. One-Dimensional Gel Electrophoresis

In order to compare venom proteomes between species, 1D gradient gels were run under both reducing and non-reducing conditions using the manufacturer (BioRad, Brisbane, Australia) protocol. Gels were prepared as follows: 0.05 mL Milli-Q H2O, 2.5 mL 30% acrylamide mix, 1.5 mL 1.0 M Tris-HCl, pH 8.45, 0.48 glycerol, 20 μL 10% APS, 2 μL TEMED (spreading gel); 0.76 mL Milli-Q H2O,

0.76. mL 30% acrylamide mix, 0.76 mL 1.0 M Tris-HCl, pH 8.45, 15 μL 10% APS, 2 μL TEMED (spacer gel); 1.56 mL Milli-Q H_2O, 0.34 mL 30% acrylamide mix, 0.63 mL 1.0 M Tris-HCl, pH 8.45, 15 μL 10% APS, 2 μL TEMED (stacking gel). Spreading gel was cast first. After it set, the spacer gel was slowly layered atop it, and after the spacer gel set the stacking gel was layered atop it. Running buffers were: 0.2 M Tris-HCl, pH 8.9 (anode buffer); 0.1 M Tris-tricine-HCl pH 8.45. The gels were run at 100 V for three hours at room temperature. Thirty μg of venom was reconstituted in Tricine loading buffer (Bio-Rad, Brisbane, Australia) with 10 mM DTT added to provide reduce conditions. Gels were stained overnight with colloidal Coomassie brilliant blue G250 (34% methanol, 3% phosphoric acid, 170 g/L ammonium sulphate, 1 g/L Coomassie blue G250). After the staining was complete, gels were destained using ultrapure water (PURELAB Flex 2, Brisbane, Australia).

3.4. Two-Dimensional Gel Electrophoresis

In order to further investigate the proteomics variation, particularly that of isoelectric variation, 2D gels were run. Then, 0.3 mg of venom sample were solubilized in 125 μL of rehydration buffer (8 M urea, 100 mM DTT, 4% CHAPS, and 0.5% ampholytes (Biolytes pH 3–10, Bio-Rad, Brisbane, Australia)) with 0.01% bromophenol blue. The sample was mixed with shaking and centrifuged for 5 min at 4 °C, 14,000 rpm. This was done to remove any insoluble material. The supernatant was loaded onto IEF strips (ReadyStrip, non-linear pH 3–10, 7 cm IPG) (Bio-Rad, Brisbane, Australia) and left overnight for passive rehydration. Protein focusing was achieved via PROTEAN i12 IEF CELL (Bio-Rad, Brisbane, Australia). The IEF running conditions were as follows: 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h and 8000 V until 98,400 V/h. Actual current in the final step of the run varied in accordance to resistance. To each strip a constant current of 50 μA was applied. After the run IPG strips were incubated for 10 min in a reducing equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2% DTT) to reduce cysteine bonds. To alkylate reduced bonds IPG strips were further incubated for 20 min in an alkylating equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide). After rinsing with SDS-PAGE running buffer, IPG strips were positioned on top of 12% polyacrylamide gels (Protean-II Plus, 18 ×20 cm, Bio-Rad Lab, Brisbane, Australia) using 0.5% agarose. Gels were run with a current of 10 mA/per gel for 20 min followed by 20 mA/per gel for the rest of the run until the bromophenol dye front was within 0.5 cm of the base of the gel. After the run, gels were briefly washed with water and stained with 0.2% colloidal Coomassie brilliant blue G250 overnight. Water was used to remove the excess of the dye after staining was complete. Visible spots were subsequently picked from gels and digested overnight at 37 °C with the use of sequencing grade trypsin (Sigma-Aldrich, Brisbane, Australia). Afterwards gel spots were washed with MiliQ water, destained (40 mM NH_4CO_3/50% acetonitrile (ACN)) and dehydrated (100% ACN); rehydration occurs in 10 μL of 20 μg/mL TPCK trypsin with subsequent incubation at 37 °C overnight. To elute peptides following solutions were used per each spot: 20 μL of 1% formic acid (FA), followed by 20 μL of 5% ACN/0.5% FA. Collected peptides were put into MS vials and subjected to LC-MS/MS analysis.

3.5. LC-MS/MS

In order to identify the toxin types present, digested gel spots and digested whole venom (shotgun) samples were processed using an Agilent (Brisbane, Australia) Zorbax stable bond C18 column (2.1 mm by 100 mm, 1.8 µm particle size, 300 Å pore size) at a flow rate of 400 µL per minute and a gradient of 1%–40% solvent B (90% acetonitrile, 0.1% formic acid) in 0.1% formic acid over 15 min or 4 min for shotgun samples and 2D-gel spots, respectively, on a Shimadzu (Brisbane, Australia) Nexera UHPLC coupled with an AB SCIEX (Brisbane, Australia) 5600 Triple TOF mass spectrometer. MS2 spectra are acquired at a rate of 20 scans per second with a cycle time of 2.3 s and optimised for high resolution. Precursor ions were selected between 80 and 1800 *m*/*z* with a charge state of 2–5 and of an intensity of at least 120 counts per second with a precursor selection window of 1.5 Da. The isotopes within 2 Da were excluded for MS2. MS2 spectra were searched against known translated transcriptome libraries or UniProt database with Proteinpilot v4.0 (ABSciex, Brisbane, Australia) using a thorough identification search, specifying iodoacetamide as an alkylation method, trypsin digestion and allowing for biological and chemical modifications (ethanolyl C or deamidated N in particular) and amino acid substitutions, including artefacts induced by the preparation or analysis processes. This was done to maximize the identification of protein sequences. Spectra were inspected manually to eliminate false positives.

3.6. Pairwise-Estimation of dN/dS

Pairwise-estimates of dN/dS were obtained for *Heloderma* Kallikreins, CRiSPs and lethal toxins using the Codeml program of PAML package.

4. Conclusions

Due to the limited amount of previously available proteomic data, these results significantly contribute to our understanding of helodermatid lizard venoms. Despite the *H. suspectum* having been separated from all other extant species for 30 million years, the venoms have a significant overall level of similarity in regards to protein/peptide types present but with variable expression within these conserved classes. This suggesting that their venoms experienced a diversifying selection pressure different from that often governs the evolution of venom in other squamate reptiles such as snakes which display significant differences in the types of proteins expressed, even at low taxonomical levels. This variation pattern is likely a consequence of the fact that all helodermatid lizards intrinsically occupy the same ecological niche and thus feed upon the similar prey items. However, the discovery of novel components represents an exciting opportunity for biodiscovery and reinforces the basic premise that poorly investigated venomous lineages represent untapped resources of molecules with potential for utilization in drug design and development.

Supplementary Materials

Supplementary materials can be accessed at: http://www.mdpi.com/2072-6651/6/12/3582/s1.

Acknowledgments

Author Contributions

B.G.F. and I.H. conceived and designed the experiments; I.K., T.N.W.J., K.S., A.N. and B.G.F. performed the experiments; I.K, T.N.W.J., K.S., A.N. and B.G.F. analyzed the data; I.K., T.N.W.J., K.S., A.N. and B.G.F. wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Douglas, M.E.; Douglas, M.R.; Schuett, G.W.; Beck, D.D.; Sullivan, B.K. Conservation phylogenetics of helodermatid lizards using multiple molecular markers and a supertree approach. *Mol. Phylogenet. Evol.* **2010**, *55*, 153–167.
2. Reiserer, R.S.; Schuett, G.W.; Beck, D.D. Taxonomic reassessment and conservation status of the beaded lizard, *Heloderma horridum* (Squamata: Helodermatidae). *Amphib. Reptile Conserv.* **2013**, *7*, 74–96.
3. Pianka, E.R.; King, D.R. *Varanoid Lizards of the World*; Pianka, E., King, D., Eds.; Indiana University Press: Bloomington, IN, USA, 2004.
4. Beck, D.D. *Biology of Gila Monsters and Beaded Lizards*; University of California Press: Oakland, CA, USA, 2005; p. 212.
5. Venom Evolution Lab, School of Biological Sciences, the University of Queensland, St. Lucia, Queensland. Private observation, 2014.
6. Bogert, C.M.; del Campo, R.M. The gila monster and its allies. The relationships, habits, and behavior of the lizards of the family Helodermatidae. *Bull. Am. Mus. Natl. Hist.* **1956**, *109*, 1–238.
7. Bouabboud, C.F.; Kardassakis, D.G. Acute myocardial-infarction following a gila monster (*Heloderma suspectum cinctum*) bite. *West. J. Med.* **1988**, *148*, 577–579.
8. Cantrell, F.L. Envenomation by the Mexican beaded lizard: A case report. *J. Toxicol.-Clin. Toxicol.* **2003**, *41*, 241–244.
9. Hooker, K.R.; Caravati, E.M. Gila monster envenomation. *Ann. Emerg. Med.* **1994**, *24*, 731–735.
10. Miller, M.F. Gila monster envenomation. *Ann. Emerg. Med.* **1995**, *25*, 720.
11. Strimple, P.D.; Tomassoni, A.J.; Otten, E.J.; Bahner, D. Report on envenomation by a Gila monster (*Heloderma suspectum*) with a discussion of venom apparatus, clinical findings, and treatment. *Wilderness Environ. Med.* **1997**, *8*, 111–116.
12. Drucker, D.J.; Nauck, M.A. The incretin system: Glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* **2006**, *368*, 1696–1705.

13. Angulo, Y.; Escolano, J.; Lomonte, B.; Gutierrez, J.M.; Sanz, L.; Calvete, J.J. Snake venomics of Central American pitvipers: Clues for rationalizing the distinct envenomation profiles of *Atropoides nummifer* and *Atropoides picadoi*. *J. Proteome Res.* **2008**, *7*, 708–719.

14. Calvete, J.J.; Juarez, P.; Sanz, L. Snake venomics. Strategy and applications. *J. Mass Spectrom.* **2007**, *42*, 1405–1414.

15. Fry, B.G.; Wickramaratana, J.C.; Lemme, S.; Beuve, A.; Garbers, D.; Hodgson, W.C.; Alewood, P. Novel natriuretic peptides from the venom of the inland taipan (*Oxyuranus microlepidotus*): Isolation, chemical and biological characterisation. *Biochem. Biophys. Res. Commun.* **2005**, *327*, 1011–1015.

16. Fry, B.G.; Wickramaratna, J.C.; Hodgson, W.C.; Alewood, P.F.; Kini, R.M.; Ho, H.; Wuster, W. Electrospray liquid chromatography/mass spectrometry fingerprinting of *Acanthophis* (death adder) venoms: Taxonomic and toxinological implications. *Rapid. Commun. Mass Spectrom.* **2002**, *16*, 600–608.

17. Fry, B.G.; Wuster, W.; Ramjan, S.F.R.; Jackson, T.; Martelli, P.; Kini, R.M. Analysis of Colubroidea snake venoms by liquid chromatography with mass spectrometry: Evolutionary and toxinological implications. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2047–2062.

18. Gutierrez, J.M.; Sanz, L.; Escolano, J.; Fernandez, J.; Lomonte, B.; Angulo, Y.; Rucavado, A.; Warrell, D.A.; Calvete, J.J. Snake venomics of the Lesser Antillean pit vipers *Bothrops caribbaeus* and *Bothrops lanceolatus*: Correlation with toxicological activities and immunoreactivity of a heterologous antivenom. *J. Proteome Res.* **2008**, *7*, 4396–4408.

19. Lomonte, B.; Escolano, J.; Fernandez, J.; Sanz, L.; Angulo, Y.; Gutierrez, J.M.; Calvete, J.J. Snake venomics and antivenomics of the arboreal neotropical pitvipers *Bothriechis lateralis* and *Bothriechis schlegelii*. *J. Proteome Res.* **2008**, *7*, 2445–2457.

20. Mackessy, S.P. Evolutionary trends in venom composition in the western rattlesnakes (*Crotalus viridis* sensu lato): Toxicity *vs.* tenderizers. *Toxicon* **2010**, *55*, 1463–1474.

21. Salazar, A.M.; Guerrero, B.; Cantu, B.; Cantu, E.; Rodríguez-Acosta, A.; Pérez, J.C.; Galán, J.A.; Tao, A.; Sánchez, E.E. Venom variation in hemostasis of the southern Pacific rattlesnake (*Crotalus oreganus helleri*): Isolation of hellerase. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2009**, *149*, 307–316.

22. Sanz, L.; Escolano, J.; Ferretti, M.; Biscoglio, M.J.; Rivera, E.; Crescenti, E.J.; Angulo, Y.; Lomonte, B.; Gutierrez, J.M.; Calvete, J.J. Snake venomics of the South and Central American Bushmasters. Comparison of the toxin composition of *Lachesis muta* gathered from proteomic *versus* transcriptomic analysis. *J. Proteomics* **2008**, *71*, 46–60.

23. Sanz, L.; Gibbs, H.L.; Mackessy, S.P.; Calvete, J.J. Venom proteomes of closely related *Sistrurus rattlesnakes* with divergent diets. *J. Proteome Res.* **2006**, *5*, 2098–2112.

24. Tashima, A.K.; Sanz, L.; Camargo, A.C.; Serrano, S.M.; Calvete, J.J. Snake venomics of the Brazilian pitvipers *Bothrops cotiara* and *Bothrops fonsecai*. Identification of taxonomy markers. *J. Proteomics* **2008**, *71*, 473–485.

25. Wagstaff, S.C.; Sanz, L.; Juarez, P.; Harrison, R.A.; Calvete, J.J. Combined snake venomics and venom gland transcriptomic analysis of the ocellated carpet viper, *Echis ocellatus*. *J. Proteomics* **2009**, *71*, 609–623.

26. Boldrini-Franca, J.; Correa-Netto, C.; Silva, M.M.; Rodrigues, R.S.; de la Torre, P.; Perez, A.; Soares, A.M.; Zingali, R.B.; Nogueira, R.A.; Rodrigues, V.M.; *et al*. Snake venomics and antivenomics of *Crotalus durissus* subspecies from Brazil: Assessment of geographic variation and its implication on snakebite management. *J. Proteomics* **2010**, *73*, 1758–1776.

27. Castro, E.N.; Lomonte, B.; del Carmen Gutiérrez, M.; Alagón, A.; Gutiérrez, J.M. Intraspecies variation in the venom of the rattlesnake *Crotalus simus* from Mexico: Different expression of crotoxin results in highly variable toxicity in the venoms of three subspecies. *J. Proteomics* **2013**, *87*, 103–121.

28. Daltry, J.C.; Wuster, W.; Thorpe, R.S. Diet and snake venom evolution. *Nature* **1996**, *379*, 537–540.

29. Forstner, M.; Hilsenbeck, R.; Scudday, J. Geographic variation in whole venom profiles from the mottled rock rattlesnake (*Crotalus lepidus lepidus*) in Texas. *J. Herpetol.* **1997**, *31*, 277–287.

30. French, W.J.; Hayes, W.K.; Bush, S.P.; Cardwell, M.D.; Bader, J.O.; Rael, E.D. Mojave toxin in venom of *Crotalus helleri* (Southern Pacific Rattlesnake): Molecular and geographic characterization. *Toxicon* **2004**, *44*, 781–791.

31. Sunagar, K.; Undheim, E.A.; Scheib, H.; Gren, E.C.; Cochran, C.; Person, C.E.; Koludarov, I.; Kelln, W.; Hayes, W.K.; King, G.F.; *et al*. Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): Biodiscovery, clinical and evolutionary implications. *J. Proteomics* **2014**, *99*, 68–83.

32. Calvete, J.J.; Fasoli, E.; Sanz, L.; Boschetti, E.; Righetti, P.G. Exploring the venom proteome of the western diamondback rattlesnake, *Crotalus atrox*, via snake venomics and combinatorial peptide ligand library approaches. *J. Proteome Res.* **2009**, *8*, 3055–3067.

33. Lopez-Lozano, J.L.; de Sousa, M.V.; Ricart, C.A.; Chavez-Olortegui, C.; Flores Sanchez, E.; Muniz, E.G.; Buhrnheim, P.F.; Morhy, L. Ontogenetic variation of metalloproteinases and plasma coagulant activity in venoms of wild *Bothrops atrox* specimens from Amazonian rain forest. *Toxicon* **2002**, *40*, 997–1006.

34. Mackessy, S.P. Venom ontogeny in the Pacific rattlesnakes *Crotalus viridis helleri* and *C. v. oreganus. Copeia* **1988**, *1988*, 92–101.

35. Brust, A.; Sunagar, K.; Undheim, E.A.; Vetter, I.; Yang, D.C.; Casewell, N.R.; Jackson, T.N.; Koludarov, I.; Alewood, P.F.; Hodgson, W.C.; *et al*. Differential evolution and neofunctionalization of snake venom metalloprotease domains. *Mol. Cell. Proteomics* **2013**, *12*, 651–663.

36. Casewell, N.R.; Wuster, W.; Vonk, F.J.; Harrison, R.A.; Fry, B.G. Complex cocktails: The evolutionary novelty of venoms. *Trends Ecol. Evol.* **2013**, *28*, 219–229.

37. Fry, B.G.; Scheib, H.; van der Weerd, L.; Young, B.; McNaughtan, J.; Ramjan, S.F.; Vidal, N.; Poelmann, R.E.; Norman, J.A. Evolution of an arsenal: Structural and functional diversification of the venom system in the advanced snakes (Caenophidia). *Mol. Cell. Proteomics* **2008**, *7*, 215–246.

38. Fry, B.G.; Wuster, W.; Kini, R.M.; Brusic, V.; Khan, A.; Venkataraman, D.; Rooney, A.P. Molecular evolution and phylogeny of elapid snake venom three-finger toxins. *J. Mol. Evol.* **2003**, *57*, 110–129.

39. Gibbs, H.L.; Mackessy, S.P. Functional basis of a molecular adaptation: Prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* **2009**, *53*, 672–679.

40. Pawlak, J.; Mackessy, S.P.; Sixberry, N.M.; Stura, E.A.; le Du, M.H.; Menez, R.; Foo, C.S.; Menez, A.; Nirthanan, S.; Kini, R.M. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB J.* **2009**, *23*, 534–545.

41. Sunagar, K.; Johnson, W.E.; O'Brien, S.J.; Vasconcelos, V.; Antunes, A. Evolution of CRISPs associated with toxicoferan-reptilian venom and mammalian reproduction. *Mol. Biol. Evol.* **2012**, *29*, 1807–1822.

42. Ali, S.A.; Jackson, T.N.; Casewell, N.R.; Low, D.H.; Rossi, S.; Baumann, K.; Fathinia, B.; Visser, J.; Nouwens, A.; Hendrikx, I.; Jones, A.; Fry, B.G. Extreme venom variation in Middle *Eastern vipers*: A proteomics comparison of *Eristicophis macmahonii*, *Pseudocerastes fieldi* and *Pseudocerastes persicus*. *J. Proteomics* **2014**, doi:10.1016/j.jprot.2014.09.003.

43. Fry, B.G.; Roelants, K.; Winter, K.; Hodgson, W.C.; Griesman, L.; Kwok, H.F.; Scanlon, D.; Karas, J.; Shaw, C.; Wong, L.; *et al*. Novel venom proteins produced by differential domain-expression strategies in beaded lizards and gila monsters (genus *Heloderma*). *Mol. Biol. Evol.* **2010**, *27*, 395–407.

44. Fry, B.G.; Winter, K.; Norman, J.A.; Roelants, K.; Nabuurs, R.J.; van Osch, M.J.; Teeuwisse, W.M.; van der Weerd, L.; McNaughtan, J.E.; Kwok, H.F.; *et al*. Functional and structural diversification of the Anguimorpha lizard venom system. *Mol. Cell. Proteomics* **2010**, *9*, 2369–2390.

45. Beck, D.D. Ecology and behavior of the gila monster in southwestern Utah. *J. Herpetol.* **1990**, *24*, 54–68.