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Intraspecific venom variation in southern African scorpion species of the genera *Parabuthus*, *Uroplectes* and *Opistophthalmus* (Scorpiones: Buthidae, Scorpionidae)

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ABSTRACT

Scorpion venoms comprise cocktails of proteins, peptides, and other molecules used for immobilizing prey and deterring predators. The composition and efficacy of scorpion venoms appears to be taxon-specific due to a coevolutionary arms race with prey and predators that adapt at the molecular level. The taxon-specific components of scorpion venoms can be used as barcodes for species identification if the amount of intraspecific variation is low and the analytical method is fast, inexpensive and reliable. The present study assessed the extent of intraspecific variation in newly regenerated venom collected in the field from geographically separated populations of four southern African scorpion species: three buthids, *Parabuthus granulatus* (Ehrenberg, 1831), *Uroplectes otjimbinguensis* (Karsch, 1879), and *Uroplectes planimanus* (Karsch, 1879), and one scorpionid, *Opistophthalmus carinatus* (Peters, 1861). Although ion signal patterns were generally similar among venom samples of conspecific individuals from different populations, MALDI-TOF mass spectra in the mass range *m/z* 700–10,000 revealed only a few ion signals that were identical suggesting that species identification based on simple venom mass fingerprints (MFPs) will be more reliable if databases contain data from multiple populations. In general, hierarchical cluster analysis (HCA) of the ion signals in mass spectra was more reliable for species identification than counts of mass-identical substances in MFPs. The statistical approach revealed conclusive information about intraspecific diversity. In combination with a comprehensive database of MALDI-TOF mass spectra in reflectron mode, HCA may offer a method for rapid species identification based on venom MFPs.

1. Introduction

Scorpion venoms comprise cocktails of proteins, peptides, and other molecules used for immobilizing prey and deterring predators (Casewell et al., 2013; Morgenstern and King, 2013). The composition and efficacy of scorpion venoms appear to be taxon-specific due to a coevolutionary arms race with prey and predators that adapt at the molecular level (Zhang et al., 2015; Zhijian et al., 2006). The taxon-specific components of scorpion venoms can be used as barcodes for species identification if the amount of intraspecific variation is low and the analytical method is fast, inexpensive and reliable.

Venom barcoding is a promising and rather simple tool for species identification (Borges et al., 2006; Debont et al., 1998; Dyason et al., 2002; Newton et al., 2007; Pimenta et al., 2003; Schaffrath and Predel, 2014; Schwartz et al., 2008). Whereas Orbitrap/ESI-MS analyses of venom samples result in unsurpassed information regarding number, molecular masses and sequences of venom peptides (Favreau et al., 2006), mass fingerprints (MFPs) obtained by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry are cheaper and more efficient. MFPs offer a useful chemotaxonomic tool if mass spectra reveal sufficient diagnostic information for the differentiation of taxa, i.e., if interspecific variation exceeds intraspecific variation.

However, venom composition, even among genetically identical individuals, may vary depending on the age of animals, time since the previous venom injection, method of venom collection, and other factors. Such variation was described in diverse venomous

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taxa (Abdel-Rahman et al., 2011; Malina et al., 2017), including scorpions (Inceoglu et al., 2003; Newton et al., 2007; Pimenta et al., 2003; Rodriguez-Ravelo et al., 2013). Therefore, the reproducibility of mass fingerprints may be more affected by natural variation than by mass spectrometry instrumentation (Favreau et al., 2006). The problem of variation between the venom composition of old vs. newly regenerated venoms (see Pimenta et al., 2003) can be minimized by collecting only newly generated venom after repeated electrical stimulation of the telson (Schaffrath and Predel, 2014). This method of venom collection should facilitate the reproducibility of mass fingerprints even if venom is collected in the field. The present study assessed the extent of intraspecific variation in newly regenerated venom collected in the field from geographically separated populations of four southern African scorpion species: three buthids, *Parabuthus granulatus* (Ehrenberg, 1831), *Uroplectes otjimbinguensis* (Karsch, 1879), and *Uroplectes planimanus* (Karsch, 1879), and one scorpionid, *Opistophthalmus carinatus* (Peters, 1861).

2. Material and methods

2.1. Venom collection and storage

Samples of the four scorpion species were collected in Angola, Botswana and Namibia (Fig. 1; Table 1). Voucher specimens are deposited in the American Museum of Natural History (New York, U.S.A.). The venom of adult scorpions of both sexes was collected in the field by electrical stimulation according to a protocol presented by Schaffrath and Predel (2014). Transcutaneous electrical stimulation was performed with a pulse width of 200–250 μs and a pulse rate of 60–130 Hz. The venom of each individual was collected into a glass capillary and subsequently transferred into a 0.5 ml microtube (Eppendorf, Hamburg, Germany) containing 200 μl 35% ethanol/0.1 TFA. Diluted venom samples were stored at 4 °C. Only newly regenerated venom, collected 24 h after the first electrical stimulation of the telson, was analyzed. The extent of variation in venom samples collected with the same methods after the first electrical stimulation, 24 h later, and seven days later was tested under laboratory conditions using *Parabuthus villosus* (Peters, 1862) from Uis, Namibia, and *U. planimanus*, from 30 km NE of Otjiwarongo, Namibia.

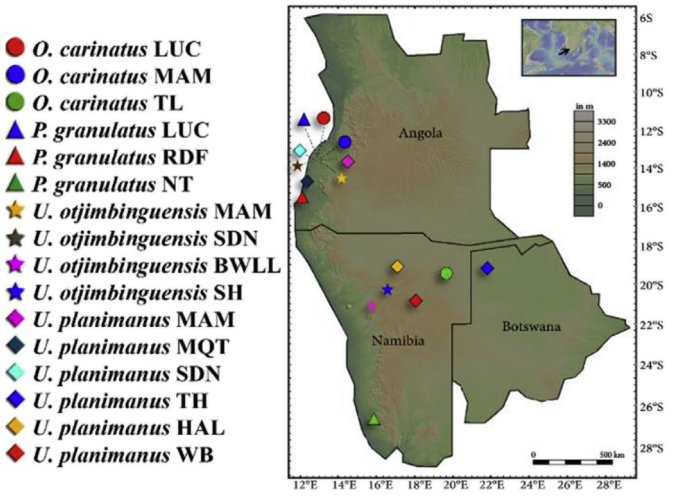

Fig. 1. Collection localities of four southern African scorpion species, *Opistophthalmus carinatus* (Peters, 1861) (circles), *Parabuthus granulatus* (Ehrenberg, 1831) (triangles), *Uroplectes otjimbinguensis* (Karsch, 1879) (stars), and *Uroplectes planimanus* (Karsch, 1879) (diamonds) analyzed in the present study. Colors represent different populations of conspecifics. Codes and corresponding GPS data are listed in Table 1. Topographic map adapted from Global Multi-Resolution Topography synthesis (Ryan et al., 2009).

Table 1
Collection localities of four southern African scorpion species, *Parabuthus granulatus* (Ehrenberg, 1831), *Uroplectes otjimbinguensis* (Karsch, 1879), *Uroplectes planimanus* (Karsch, 1879), and *Opistophthalmus carinatus* (Peters, 1861). Coordinates in decimal degrees for the WGS84 datum.

Country	Locality	Code	Latitude	Longitude	Altitude
Angola	Lucira	LUC	−13.646	12.619	374 m
Angola	Mamué	MAM	−13.799	13.118	639 m
Angola	Mariquita	MQT	−14.771	12.386	289 m
Angola	Rio dos Flamingo	RDF	−15.592	12.194	130 m
Angola	Serra de Neve	SDN	−13.695	12.922	462 m
Botswana	Tsodilo Hills	TH	−18.785	21.739	1002 m
Namibia	Brandberg	BWLL	−21.023	14.682	464 m
Namibia	Halali	HAL	−19.039	16.470	1112 m
Namibia	Tirasberge	NT	−26.189	16.353	1005 m
Namibia	Sophienhöhe	SH	−20.119	16.059	1330 m
Namibia	Tsumkwe	TL	−19.444	19.756	1219 m
Namibia	Waterberg	WB	−20.478	17.303	1460 m

2.2. MALDI-TOF MS analysis

0.3 μl of 10 mg/ml 2,5-dihydroxybenzoic acid (Sigma-Aldrich, Steinheim, Germany), dissolved in 20% acetonitrile/1% formic acid, was used as a matrix and mixed with the same quantity of stored venom directly on the sample plate for MALDI-TOF mass spectrometry before air drying at room temperature. MALDI-TOF mass spectrometry was performed in reflectron positive ion mode on an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). Samples were recorded in the mass ranges of *m/z* 700–4000 and *m/z* 4000–10,000. Intensity and number of laser shots were adjusted to obtain an optimal signal-to-noise ratio; most data resulted from 3000 shots at a laser frequency of 666 Hz. Bruker peptide and protein standard kits were used for calibration. MS/MS experiments were conducted using Bruker LIFT™ technology implemented in the UltrafleXtreme or ABI 4800 proteomics analyzer (Applied Biosystems, Framingham, U.S.A.) in gas-off and gas-on (assignment of Leu/Ile ambiguities) mode. Peptide sequences were identified by manual analysis of fragment ions and subsequent comparison of predicted (http://prospector.ucsf.edu) and experimentally-obtained fragment patterns.

2.3. Data analysis

MFPs were processed with FlexAnalysis 3.0 (Bruker Daltonik, Bremen, Germany). Only ion signals with a minimum intensity of 3% were manually selected for subsequent analysis. Ion signals uniquely obtained in all individuals from a single population or a single species were considered species-specific or population-specific markers, respectively. The percentage of population-specific signals was calculated for each population. In this context, the average number of ion signals in MFPs represented the initial value of 100%. Matrix signals and signals from multiple charged ions were excluded. Similarity analysis of the binary mass list was performed using Dice's coefficient. This similarity analysis provided the basis for a single linkage agglomerative hierarchical clustering analysis (HCA). HCA was performed with MATLAB R2017a (MathWorks, MA, U.S.A.).

3. Results and discussion

3.1. Comparison of MFPs of venom samples collected from the same individuals immediately after the first electrical stimulation, 24 h later, and seven days later

Recent analyses have shown that venom composition may differ between freshly regenerated venom and venom from the first extraction (Schaffrath and Predel, 2014). These differences appear to be more obvious in the scorpionid taxa with large pedipalps which seem to use their stinger less regularly than buthid species with rather weak pedipalps (S. Schaffrath, unpublished results; see also Casper, 1985). We used two individuals of *P. villosus* and *U. planimanus* under laboratory conditions to verify that an interval of 24 h after the first electrical stimulation is sufficient to obtain reproducible venom MFPs in buthid scorpions. Both individuals had their last meal 14 days before the first venom extraction. The presence of ion signals was very similar in samples collected immediately after the first electrical stimulation, 24 h later, and seven days later (Figs. 2 and 3). All subsequent venom extractions were performed 24 h after a first electrical stimulation of the telson in the field.

3.2. Comparison of venom MFPs from single individuals of four southern African scorpion species

Venom compounds collected in the field from single individuals enabled the different scorpion species to be clearly identified (Fig. 4). Most of the ion signals observed were specific to a single species only. Only individuals of the two species of *Uroplectes* shared several mass-identical substances. These results were expected, because a separation of southern African *Parabuthus* species with MALDI-TOF MFPs was previously reported (Dyason et al., 2002). However, the species-specific venom code for *P. granulatus* reported by Dyason et al. (2002), which covered only three putative Na^+ channel toxins in the mass range of *m/z* 6500–7,500, did not match the data presented here for the same species. A single possible hit may refer to another species, *P. kalaharicus*, when converting the listed average masses in monoisotopic masses, but this match could be coincidental. Two conclusions can be drawn from these results. First, the published venom code for *Parabuthus* species is not species-specific but rather population-specific (exact collection localities were not given by Dyason et al., 2002). Second, a wider mass range should be evaluated to search for species-specific components in the MFPs. MFPs of several conspecific individuals from the same locality (i.e., population) were initially compared to address this question. Conspecific individuals from different populations were then analyzed in the same manner to estimate geographic variation and constancy in the MFPs of each scorpion species.

3.3. Comparison of venom MFPs obtained from multiple conspecific individuals collected at the same localities

Previous work reported sex-related differences as a potential source of variation in the venom of scorpions. Some of these studies reported variation in a mass range not analyzed in the present study (Abdel-Rahman et al., 2009) or suggested quantitative variation in the venom complements (Miller et al., 2016; Schwartz et al., 2008). In the present study, only qualitative differences (presence/absence of ion signals) were recorded. Different ion intensities of otherwise mass-identical ion signals were not considered to be variation. Qualitative differences in venom compounds observed among males and females were, however, reported for ion signals in a mass range around m/z 5000 by De Sousa et al. (2010) and Yamaji et al. (2004). Own experiments did not reveal significant differences among the venom MFPs of male and female *Heterometrus cyaneus* (C.L. Koch, 1836) (Scorpionidae) under laboratory conditions (Schaffrath, unpublished). In addition, a comparison of the MFPs of male and female *U. planimanus* sampled in the field in Botswana and Namibia did not show sex-related differences. Therefore, the MFPs of different individuals collected at the same localities (see Fig. 5) were compared to assess the variation among MFPs within a single population; regardless of sex. Variation within a single population was observed among four individuals of *P. granulatus* from Angolan locality RDF (Fig. 5). In the

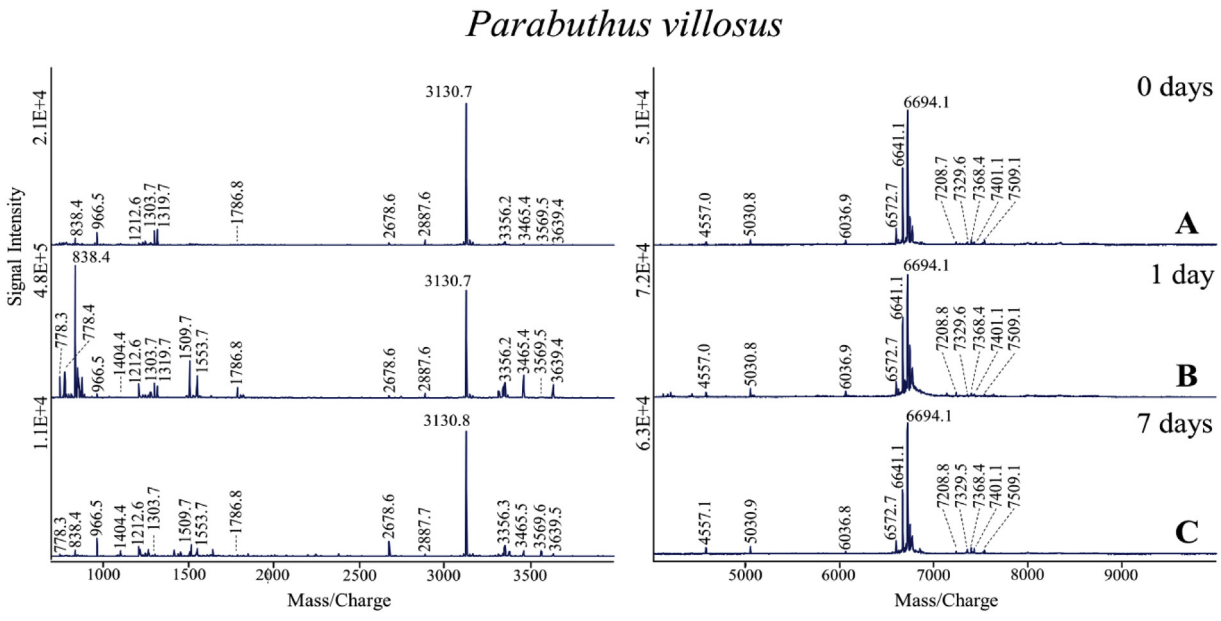

Fig. 2. Comparison of three venom extractions from *Parabuthus villosus* (Peters, 1862) (left panel m/z 700–4000; right panel m/z 4000–10,000). Prominent ion signals are labeled. A) Extracted venom from the first electrical stimulation. B) Second venom extraction 24 h later. C) Third venom extraction seven days later. Despite variation in the relative abundance of some ion signals, the presence of ion signals (yes/no) did not change.

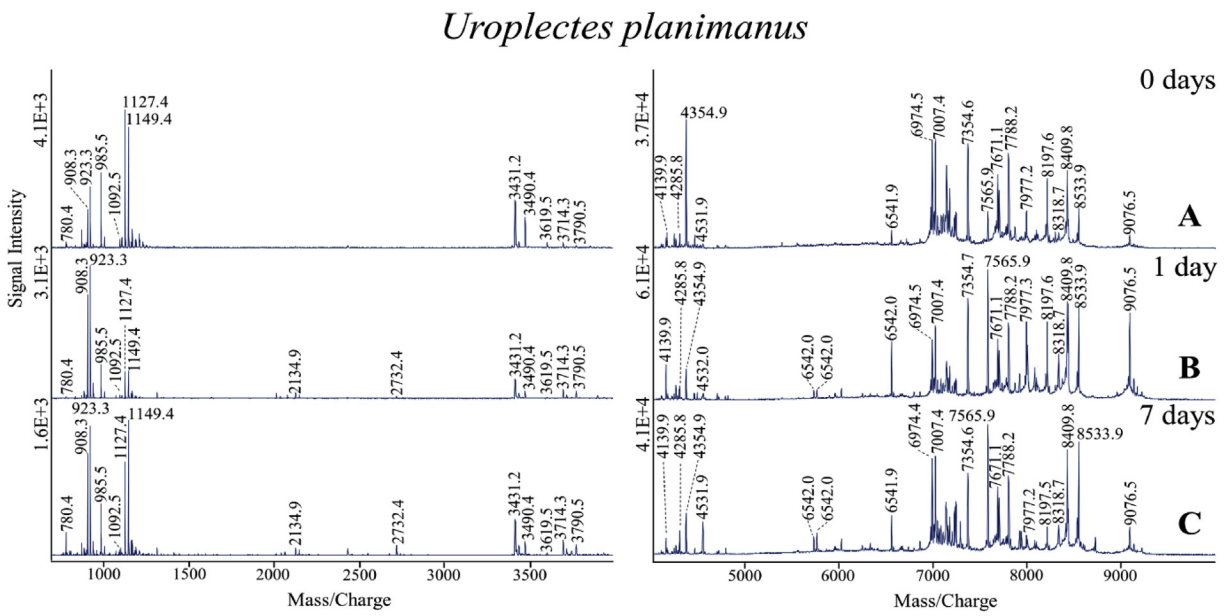

Fig. 3. Comparison of venom fingerprints from three venom extractions of *Uroplectes planimanus* (Karsch, 1879) (left panel m/z 700–4000; right panel m/z 4000–10,000). Prominent ion signals are labeled. A) Extracted venom extraction from the first electrical stimulation. B) Second venom extraction 24 h later. C) Third venom extraction seven days later. Despite variation in the relative abundance of some ion signals, the presence of ion signals (yes/no) did not change.

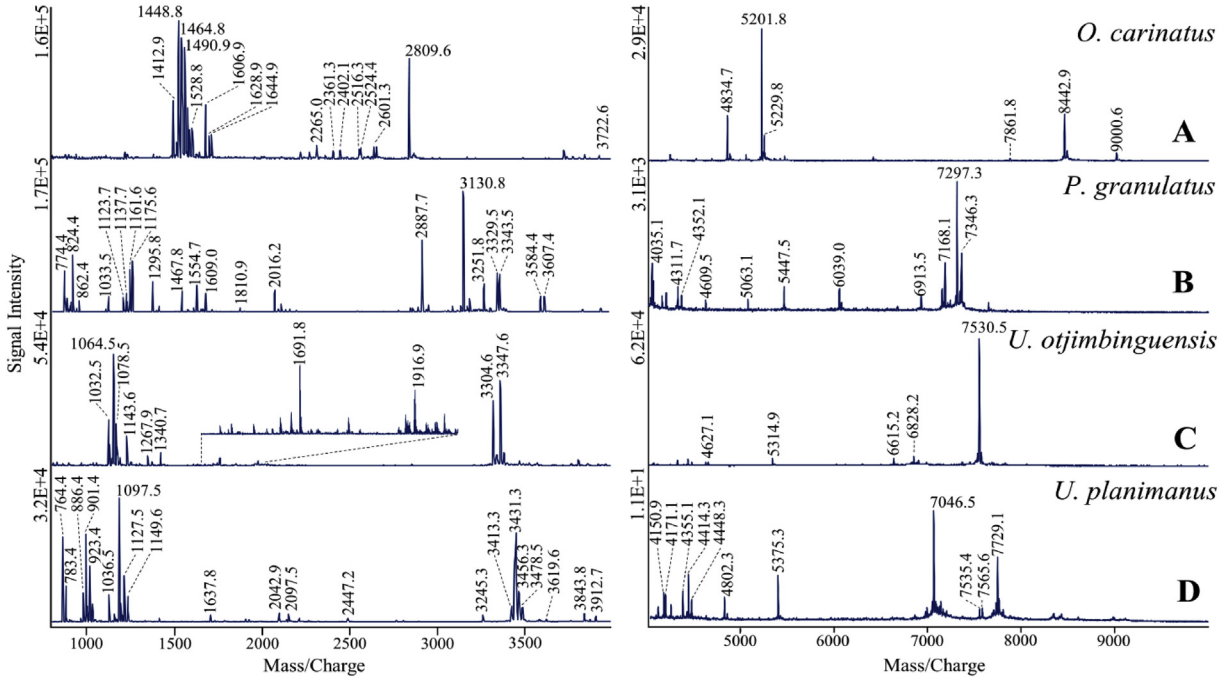

Fig. 4. Comparison of MALDI-TOF MFPs of diluted venom samples collected in the field from four southern African scorpion species. MFPs are specific for each sample. Two mass ranges (left panel m/z 700–4000; right panel m/z 4000–10,000) were recorded separately. Prominent ion signals are labeled. A) *Opistophthalmus carinatus* (Peters, 1861), TL; B) *Parabuthus granulatus* (Ehrenberg, 1831), RDF; C) *Uroplectes otjimbinguensis* (Karsch, 1879), SH; D) *Uroplectes planimanus* (Karsch, 1879), HAL.

complete mass range of *m/z* 700–10,000, an average of 58 distinct ion signals was recorded among the individual MFPs; 44 of these signals were in the mass range of *m/z* 700–4000. Only ion signals occurring in all MFPs were considered typical for a population and divergent MFPs were not eliminated a posteriori. Among the *P. granulatus* individuals from locality RDF, 35 ion signals were observed in all MFPs, i.e., 60% of the average number of ion signals obtained in each of the four MFPs. This does not imply that only 60% of the observed ion signals represent compounds common to all individuals within the population. It reflects those ions detected in all venom MFPs from a single population. Variation among the ion signals increased in the higher mass range and included the putative sodium channel modulators.

The same approach was repeated using the individuals of all populations ($n = 60$) of the four scorpion species (Table 2; Supplementary Material 1). The average number of ion signals

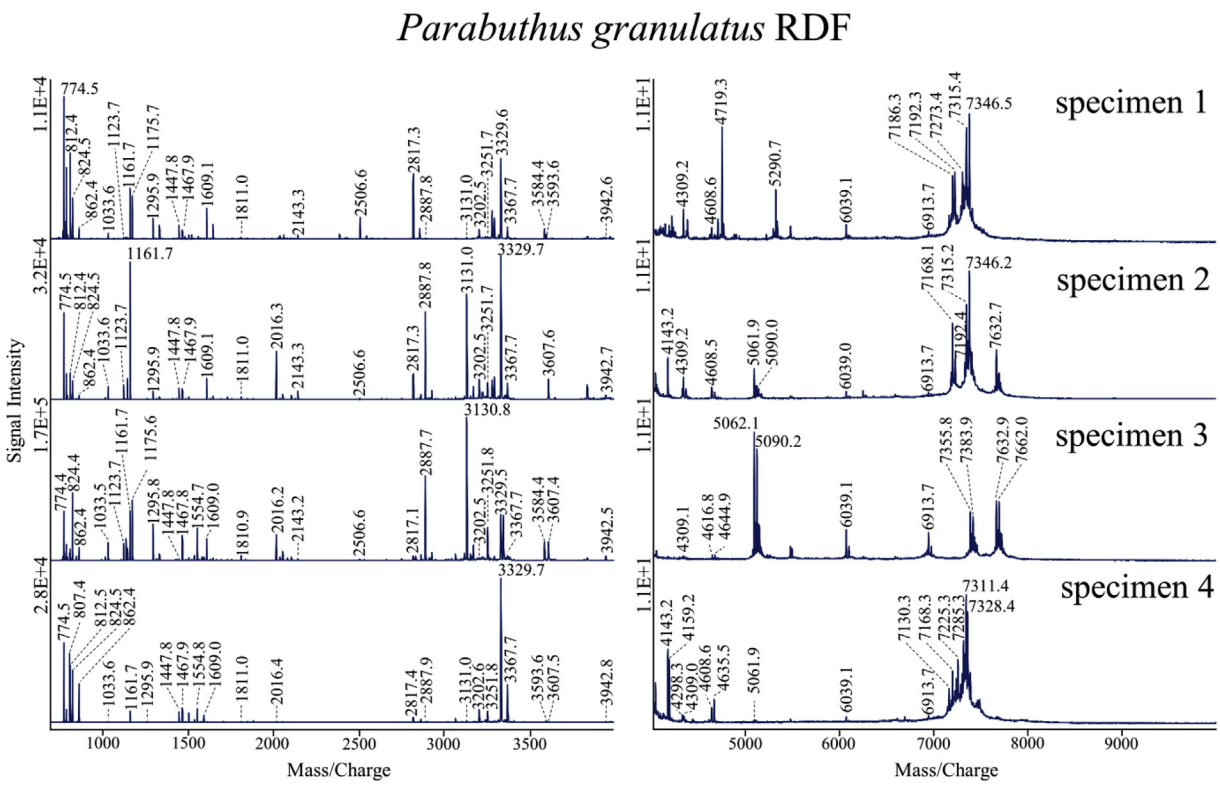

Fig. 5. Comparison of MALDI-TOF MFPs of four specimens of the southern African scorpion, *Parabuthus granulatus* (Ehrenberg, 1831) from Angolan locality RDF (left panel m/z 700–4000; right panel m/z 4000–10,000). Prominent ion signals are labeled. Ion signal identities decrease in the higher mass range.

Table 2
Number of specimens (*n*) of four southern African scorpion species, *Parabuthus granulatus* (Ehrenberg, 1831), *Uroplectes otjimbinguensis* (Karsch, 1879), *Uroplectes planimanus* (Karsch, 1879), and *Opistophthalmus carinatus* (Peters, 1861) analyzed per locality, number of ion signals in MFPs, and percentage of ion signals present in MFPs of all conspecific individuals from each locality. Percentage calculated using average number of ion signals in MFPs of different individuals from each locality. Note decrease of ion signals among conspecific individuals from each population in the higher mass range (*m/z* 4000–10,000).

Species	Code	*n*	Ion signals	Ion signals all MFPs	%	%<4000	%>4000
Opistophthalmus carinatus	LUC	4	24–26	17	67.3	76.5	23.5
	MAM	3	23–25	19	80.3	73.7	26.3
	TL	4	29–40	25	70.9	84.0	16.0
Parabuthus granulatus	LUC	3	34–54	26	61.9	84.6	15.4
	RDF	4	56–65	35	59.3	88.6	11.4
	NT	2	45	40	88.9	87.5	12.5
Uroplectes otjimbinguensis	MAM	2	35	30	85.7	90.0	10.0
	SDN	4	36–37	19	52.8	94.7	5.3
	BWLL	4	33–46	17	44.7	70.5	29.5
	SH	5	38–48	28	65.1	89.3	10.7
Uroplectes planimanus	MAM	4	56–73	40	64.0	75.0	25.0
	MQT	3	51–69	23	37.7	100.0	0.0
	SDN	4	68–76	38	54.1	78.9	21.1
	TH	5	24–41	15	47.2	73.3	26.7
	HAL	4	47–59	29	55.8	79.3	20.7
	WB	5	46–59	23	44.1	73.9	26.1

observed in MFPs varied considerably but, in most conspecific populations, 50–70% of ion signals were observed in MFPs of all individuals analyzed. In general, the signals common to all conspecific individuals collected from the same locality (population) decreased in the higher mass range. The list of ion signals shared by all conspecific individuals from the same population (Supplementary Material 2) was used in subsequent analyses to detect variation among populations. This list also includes potentially species-specific ion signals.

3.4. Comparison of venom MFPs obtained from conspecific individuals collected at different localities

In order to discriminate among population-specific and species-specific ion signals, the ion signals typical of each species were identified from the data compiled for the different conspecific populations (Supplementary Material 2). Only four ion signals (ca. 9% of the average number of ion signals obtained in single MFPs) were found in all mass spectra of the individuals from different

populations of *P. granulatus*. These signals were detected in the low mass range of *m/z* 750–900. At least two of these substances represent peptides (*R*FYPSR-NH_2, *R*FIPSR-NH_2; Fig. 6) and exhibit sequence similarity to recently described $NPDB_{21}$, new-type peptides without disulfide bridges, and with a GKR processing signal in the precursor (Zhong et al., 2017). The three ion signals mentioned as a species-specific code in Dyason et al. (2002) were not detected in any of the MFPs from *P. granulatus* venom samples analyzed for the present study. The number of potentially species-specific ion signals does not necessarily decrease as more individuals or populations are analyzed. In the case of *U. planimanus*, 25 individuals from six populations were compared, and five ion signals, including putative potassium channel modulators, were shared among them. In this species, one of the commonly occurring shorter peptides was fragmented, yielding the sequence of an unknown histidine-rich peptide (GDKHHHGPED-NH_2).

The potentially species-specific ion signals of the four scorpion species investigated in the present study are summarized in Table 3. These signals do not cluster in a mass range typical of venom components like antimicrobial, hemolytic, immunemodulating, antifungal or hormone-like peptides (Lee et al., 2004; Pimenta and De Lima, 2005; Ramirez-Carreto et al., 2012, 2015; Zeng et al., 2005), potassium channel modulators (*m/z* 3000–4500 Da; (Lima and Martin-Eauclaire, 1995; Pimenta et al., 2001; Rodriguez de la Vega and Possani, 2004; Tytgat et al., 1999) or sodium channel modulators (*m/z* 6500–8000; (Batista et al., 2004; Rodriguez de la Vega and Possani, 2005). Considering the documented variation among the venom composition of different conspecific populations, it seems possible that the number of species-specific ion signals will further decrease as more populations are analyzed. However, the widespread *P. granulatus* was analyzed along a transect of nearly 2000 km and it is likely that MFPs from additional populations will overlap MFPs from populations analyzed in the present study. These results suggest that species identification based on simple MFPs will be more reliable if databases contain information from multiple conspecific populations rather than a few species-specific ion signals.

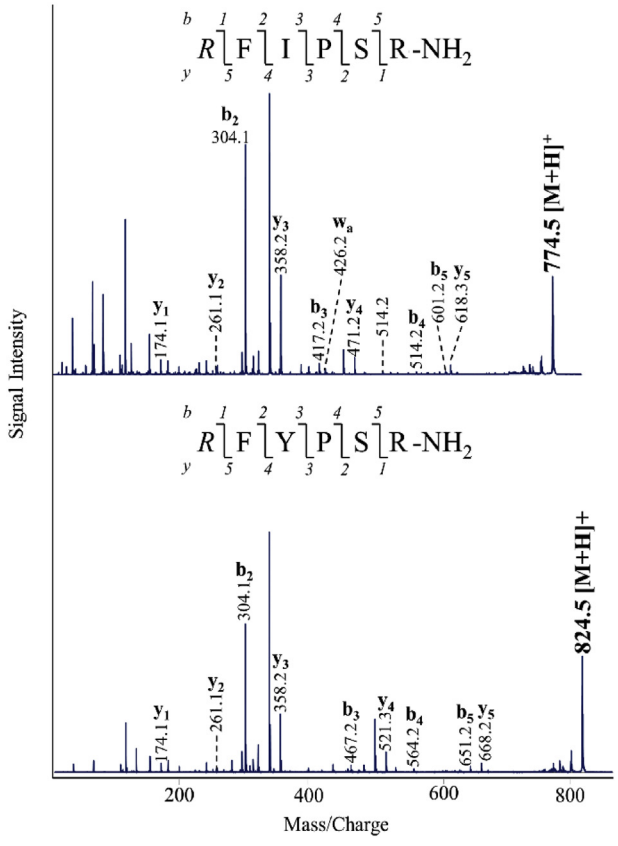

Fig. 6. MSMS spectra of two related peptides observed in MFPs of all specimens of the southern African scorpion, *Parabuthus granulatus* (Ehrenberg, 1831) analyzed in the present study. Prominent b- and y-ions are labeled. Mass spectra do not differentiate between N-terminal R or GV. Sequences resemble recently described new-type peptides without disulfide bridges and with GKR processing signal in the precursor ($NPDB_{21}$) (Zhong et al., 2017).

Table 3
Average number of potentially species-specific ion signals observed in MFPs compared to percentage of ion signals present in MFPs of all individuals of four southern African scorpion species, *Parabuthus granulatus* (Ehrenberg, 1831), *Uroplectes otjimbinguensis* (Karsch, 1879), *Uroplectes planimanus* (Karsch, 1879), and *Opistophthalmus carinatus* (Peters, 1861).

Species	% average ion signals in all conspecific specimens	Ion signals in all conspecific specimens
Opistophthalmus carinatus	19%	1412.8
		1490.9
		1528.8
		1606.9
		2589.5
		2601.3
Parabuthus granulatus	9%	774.5
		812.4
		824.5
		862.4
Uroplectes otjimbinguensis	5%	1143.6
		1149.6
Uroplectes planimanus	9%	1092.58
		1127.53
		1149.53
		3490.46
		3619.62

The percentage of species-specific ion signals in MFPs of different conspecific populations varies among the four genera studied (Table 3). The least congruence was observed among the *U. otjimbinguensis* populations. One possible reason is the small size of these scorpions which might result in a low abundance of several venom components that are present but not clearly detectable in MFPs. Nevertheless, a detailed analysis of the MFPs among populations of *U. otjimbinguensis* and *U. planimanus* indicated that populations which are geographically proximate to one other share more similarities. In addition, more congruence was observed among individuals collected along a West–East transect (northern Namibia–northeast Botswana) than along a South–North transect (northern Namibia–southern Angola). These differences suggest possible vicariance between populations rather than technical problems during venom extraction. Northern Namibia is separated from southern Angola by the Kunene River.

3.5. Hierarchical cluster analysis (HCA) of the venom fingerprints

The relevance of venom fingerprints for identification of species and even populations is illustrated by the HCA (Fig. 7). Conspecific samples grouped according to species. Moreover, the arrangement of the four species was consistent with currently accepted phylogenetic relationships among the families and genera in question (Prendini and Wheeler, 2005). *Opistophthalmus* (Scorpionidae) was placed sister to the remaining species which belong to Buthidae. Within Buthidae, the two *Uroplectes* species formed a group, sister to the species of *Parabuthus*. Even individuals of the closely related *U. otjimbinguensis* and *U. planimanus*, collected at the same localities, always clustered together. Although the number of identical

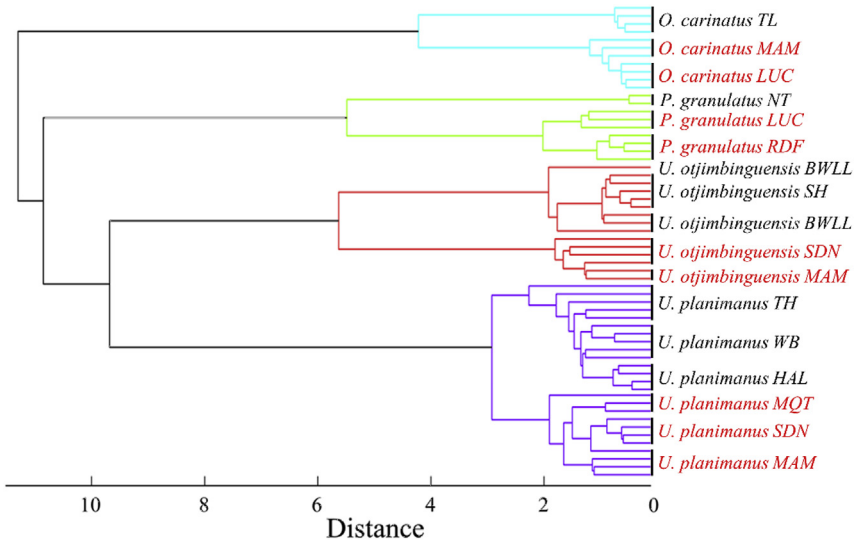

Fig. 7. Hierarchical clustering of venom fingerprint data of 60 individuals of four southern African scorpion species, *Parabuthus granulatus* (Ehrenberg, 1831), *Uroplectes otjimbinguensis* (Karsch, 1879), *Uroplectes planimanus* (Karsch, 1879), and *Opistophthalmus carinatus* (Peters, 1861), based on similarity analysis with the Dice coefficient. Distances correlate with the obtained pairwise similarities. Individuals of the different species are clearly separated from each other and the topology of the dendrogram is consistent with currently accepted phylogenetic relationships. Note that the Angolan populations of all species (red letters) are always separated from Namibian/Botswana populations.

ion signals in MFPs among different populations was rather low, the Angolan populations of all species analyzed were distinctly separated from the Namibian populations, in each case. These results suggest that species identification is more reliable with HCA than using counts of mass-identical substances in MFPs.

4. Conclusions

The use of complex venom MFPs for species identification is a widely-accepted concept and works effectively with MALDI-TOF mass spectra which are easy to obtain. As demonstrated in the present study, these spectra contain sufficient information to consistently identify species. The data presented, based on newly regenerated venom extracted in the field, confirmed considerable variation among venom components even within conspecific populations. This variation generally increased with geographical distance until few ion identical signals remained among conspecific individuals. HCA was more reliable for species identification than counts of mass-identical substances in mass fingerprints. As these statistical analyses also contain potential information about intraspecific diversity, they might offer a promising taxonomic tool that complements other methods of species identification by using ion signal identity. For this to work, multiple mass spectra from different conspecific populations will need to be included in a reference database that can be queried to reliably assign new venom samples to species by comparison with mass spectra from authoritatively identified samples.

Author contributions

Stephan Schaffrath performed experiments (including collecting and 'milking' scorpions), and data analysis; Lorenzo Prendini assisted with collecting activities in Angola and determined the species; Reinhard Predel designed the research, organized collecting activities. All authors contributed to the writing of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.toxicon.2018.02.004.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.toxicon.2018.02.004.

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