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Determination of species-specific components in the venom of *Parabuthus* scorpions from southern Africa using matrix-assisted laser desorption time-of-flight mass spectrometry

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The aim of the present study was to analyze mass spectra of scorpions belonging to the genus *Parabuthus* (Pocock 1890) by means of matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOFMS) and to construct a species-specific venom code for species identification. The venom compositions of sixteen *Parabuthus* species, occurring in southern Africa, were characterized using representative peaks in the molecular mass range of 6400–8400 Da. This mass range is characteristic for the typical long-chain neurotoxins influencing sodium channels. Only a few of these peptides have been sequenced up to now. The impetus for development of these species-specific profiles was the observation of unique, highly reproducible mass spectral peaks within a specific species. An identification label for all the different species could be found using a minimum number of peaks. MALDI-TOFMS is therefore proposed as a complementary method to morphological and behavioural characteristics for species and ultimately subspecies discrimination.

Scorpion venom is a mixture of various substances, most of which are peptide toxins known to interact with different kinds of ion channels in the membranes of excitable cells.[1,2] The most obvious symptoms of scorpion envenomation are neurological,[3] indicating that the primary targets of scorpion (neuro)toxins are ion channels in the neuronal membrane. Because of structural differences between ion channels of mammalian and insect cells some of these toxins interact specifically with either of these channels.[4,5] As a result of the specific interaction with different types of ion channels, scorpion toxins have been used as valuable tools to study the molecular characteristics and physiological functioning of these channels,[1,2,4] as well as diseases that are directly related to channel function.[6]

In general, these neurotoxins can be divided into two groups according to differential effects on K^+ and Na^+ channels. A first group has been described as long-chain peptides containing 60–70 amino acids cross-linked by four disulfide bridges. The most apparent effect of these toxins is their interaction with the gating mechanism of voltage-dependent sodium channels in excitable membranes.[1,5,7–9] The second group consists of short-chain toxins with 30–40 amino acids cross-linked by three[7,10–13] or four[14] disulfide bridges. The molecular targets of these toxins are several types of voltage-dependent potassium and chloride channels as well as calcium-activated potassium channels in a variety of cell types.[7,10–13] The short-chain toxins have been classified into twelve molecular subfamilies, containing 49 different peptides.[15] The venom of *Parabuthus* species contains various peptides interacting with potassium,[15] calcium[16] and sodium channels.[17] In addition, cationic amphipathic peptides with an intermediate length (45 amino acids), with pore-forming and antimicrobial[18] activity, have recently been isolated from *Parabuthus schlechteri* (Purcell 1899).[19] These peptides may have a function in the innate immunity of scorpions, but in addition their depolarizing effects may contribute to hyper-excitation of peripheral neurons.

Four scorpion families, *viz*. Scorpionidae (Latreille 1802), Ischnuridae (Simon 1879), Bothriuridae (Simon 1880)[20] and

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Buthidae (C.L. Koch 1837),[3,21] occur in southern Africa. The most dangerous and medically important species in the region belong to the genus *Parabuthus* of the family Buthidae. The genus *Parabuthus* currently comprises 28 species, 20 of which occur in southern Africa, and is restricted to arid and semi-arid regions. *Parabuthus granulatus* (Ehrenberg 1831) is responsible for the majority of serious envenomations, often resulting in fatality, in the Western Cape.[3] In southern Zimbabwe, where scorpion stings are common, *Parabuthus transvaalicus* (Purcell 1899) is mostly involved.[22]

MALDI-TOFMS has been used successfully in the identification of the molecular mass of a wide variety of peptides in crude venom of scorpions,[23] spiders,[24–27] snakes,[28–30] cone shells[31] and tropical ants.[32,33] Peptide mixtures are relatively easily analyzed for molecular mass at femtomole levels with an 0.01% accuracy, together with fast sample preparation.[34] It is an accurate, sensitive and convenient method that can be applied in the presence of salts and buffers[32] and is ideally suited in cases of limited venom supply.[27,35] Therefore, for analysis of small quantities of venom it has been considered as the tool of choice.[24–26]

The aim of the present study was to determine a typical venom profile of reproducible MS peaks for each of the 16 *Parabuthus* species collected in southern Africa and Namibia. Additionally, by characterization of species-specific *m/z* profiles a number of scientific achievements and applications can be expected. (1) The identification of new peptides belonging to a specific class of toxins that can be selected for purification, sequencing and testing for ion channel activity. Chemically related structures with specific ion channel activity sequenced in one species might be identified in other species by comparing their venom profiles. Since some scorpions are readily available while others are not, such a strategy can assist in the elucidation of the components of rare venoms. (2) The analysis of the stability and constancy of the venom composition that might be influenced by several factors including diet, duration of captivity and seasonality. (3) The construction of a species-specific venom code for identification of species, and eventually subspecies, within a genus.

METHODS

Collection of venom and sample preparation

Species of *Parabuthus* were collected from different locations and are summarized in Table 1. Venom was extracted by electrical stimulation of the telson with a frequency- and voltage- controlled stimulator. The released venom drops were collected in Eppendorff tubes containing 1 mL of deionized water. Venom was then lyophilized and, if not used immediately, frozen at −20 °C prior to MALDI-TOFMS analysis. To construct a venom code for species determination we selected only the most reproducible peaks in the range where the long-chain Na^+ channel toxins usually occur. The group of Na^+ toxins was used because of the high constancy of the individual patterns obtained for the relatively small quantity of animals that were available. As the aim of this study was not to obtain a functional map of all the existing peaks in the venom, but only the most reproducible peaks to provide a venom code for each species, no software algorithm was used to separate MS peaks that were superimposed. A clear inflection point on a curve was used as an indication for a separate peak. This approach may result in the loss of some peaks but, as we were only interested in a small number of prominent values for determining a venom profile, the experimental results obtained validated this approach. The reproducibility of venom component patterns was established by analyzing venoms from eight individual scorpions per species, for 14 of the 16 species. Any *m/z* values that were absent in the spectrum for more than one individual scorpion per species were excluded. Only one individual was available for *P. namibensis*, and *P. nanus* is so small that we decided to pool the venom of seven individuals in two batches. For *P. nanus* only a peak occurring in both batches, and for *P. namibensis* only the most prominent, clearly separated peaks in the MS profile were used.

MALDI-TOFMS was performed using a Voyager DE-PRO (Perseptive Biosystems) instrument. Freeze-dried venom samples contained in 2 mL Eppendorf vials were dissolved in 10–100 μL Milli-Q water depending on the quantity of

Table 1. Summary of the family, species and location of scorpions collected for this study (SA = South Africa)

Family	Species	Country/Province
Buthidae	*Parabuthus capensis* (Ehrenberg 1831)	Northern Cape, SA
	P. granulatus	
	Parabuthus kalaharicus (Lamoral 1977)	
	Parabuthus kuanyamarum (Monard 1937)	
	Parabuthus laevifrons (Simon 1888)	
	Parabuthus nanus (Lamoral 1979)	
	Parabuthus raudus (Simon 1888)	
	Parabuthus brevimanus (Thorell 1876)	
	Parabuthus planicauda (Pocock 1889)	
	P. transvaalicus	Northern Province, SA
	Parabuthus mossambicensis (Peters 1861)	
	Parabuthus stridulus (Hewitt 1913)	
	Parabuthus namibensis (Lamoral 1979)	Namibia
	Parabuthus kraepelini (Werner 1902)	
	P. schlechteri	
	Parabuthus villosus (Peters 1862)	
Scorpionidae	*Opistophthalmus wahlbergii* (Thorell 1876)	Northern Province, SA
	Opistophthalmus carinatus (Peters 1861)	Northern Cape, SA

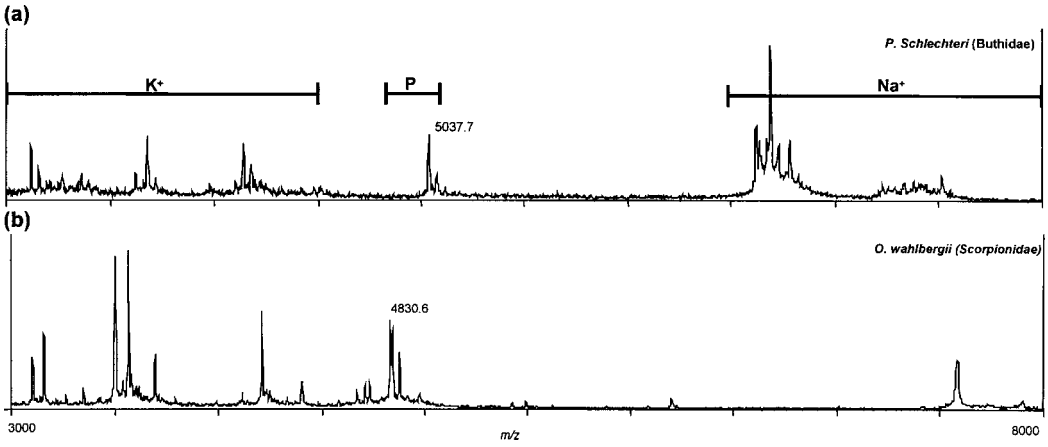

Figure 1. MALDI-TOF mass spectra of (a) *Parabuthus schlechteri* and (b) *Opistophthalmus wahlbergii* in the mass range *m/z* 3000–8000. *m/z* values related to short-chain (K^+), long-chain (Na^+) and pore-forming toxins in *Parabuthus* are indicated by K^+, Na^+ and P, respectively.

material. Further dilutions were made based on the quality of spectra and the presence of peptide dimer ions. Typically 1 μL of sample was spotted on the gold-plated MALDI sample plate together with 1 μL α-cyano-4-hydroxycinnamic acid (10 mg in 60% acetonitrile/0.3% TFA) followed by air drying. The instrument was calibrated using angiotensin I (MW 1297.51 Da) and oxidized insulin B chain (MW 3496.96 Da). All mass spectra were recorded with and without the two reference peptides as internal standard using a two-point calibration. The errors in the masses of the peptides are close to 0.05%. All spectra were recorded in the *m/z* range 1000–15000 using accelerating, grid and guide wire potentials of 20000, 19000 and 1000 Vs, respectively, and a 400 ns delayed extraction setting. Generally, 250 spectra were accumulated in the linear mode of the instrument at a 2 ns resolution.

RESULTS AND DISCUSSION

The venom composition of scorpions of southern Africa is largely unknown, and its analysis has only recently been started for some species of the *Parabuthus* genus.[15,36] Because the severity of scorpion envenomation, ranging from local pain to generalized neurological symptoms and death, varies between scorpion families, genera and species, the composition of the venom and quantity of different groups of toxins are expected to vary. Figure 1 illustrates a comparison of the mass spectral patterns for two important scorpion families in southern Africa: Buthidae, with the medically most important genus *Parabuthus* (*P. schlechteri* shown in Fig. 1 (a)), and Scorpionidae, with the genus *Opistophthalmus*[20] (*O. wahlbergii* shown in Fig. 1 (b)). Figure 1 illustrates the presence of two main groups of peaks present in the venom of *Parabuthus*: the short-chain K^+ peptides (indicated in the figure by K^+) in the 3–4.5 kDa range, and the long-chain toxins (indicated by Na^+) in the 6.5–8 kDa range. In *Parabuthus* the cluster of peaks in the mass range 6.5–8 kDa were always present in all specimens analyzed.

In the *m/z* 4830–5040 range peaks were found in the venom of some species of both families. In this range of *m/z* values peptides with pore-forming activity were found for *P. schlechteri* (*m/z* 5030[19]) and *O. carinatus*.[37] Three peaks were always present in this region for all the *P. wahlbergii* studied. Pore-forming activity in the venom of South African scorpions was first described by Badenhorst[38] in the venom of *O. carinatus*. Most probably the presence of pore-forming, membrane-disrupting peptides in crude venom is responsible for cell death of cardiac myocytes that was found in the absence of a functional sodium-calcium exchange.[39] In contrast to *P. schlechteri* these peaks could not be detected in the venom of *P. granulatus*, a scorpion with no pore-forming activity in its venom. The presence of pore-forming peptides was found to be highly variable in *P. schlechteri*.[19] Because of the evidently high reproducibility of MS peaks in the range of *m/z* values characteristic for the long-chain Na^+ channel toxins, this group was chosen as the basis for constructing the venom profiles for categorizing the 16 *Parabuthus* species. Typical α-effects that correlate with these toxins were described for *Parabuthus* by Badenhorst.[36,38,40]

Figures 2 (a)–(c) show the mass spectra of three individual *P. transvaalicus* scorpions. Although it is evident that the relative intensities of individual peaks vary, the peaks are always clustered in two main groups separated by a *m/z* range in which no peptides occur. The same figure shows the spectra of four other *Parabuthus* representatives (*P. mossambicensis*, *P. villosus, P. granulatus* and *P. kuanyamarum*). The spectra obtained for these four species are clearly different from one another. The spectra of *P. mossambicensis* (Fig. 2(d)) and *P. villosus* (Fig. 2(e)) also have two groups of peaks similar to those in the spectrum of *P. transvaalicus*, indicating that they belong to the same subgroup of *Parabuthus* scorpions although for *P. villosus* the first group and for *P. mossambicensis* the second group of peaks cover a wider *m/z* range compared to *P. transvaalicus*. The venom of *P. granulatus* (Fig. 2(f)) is an example of venom with a dominant second group of peaks and *P. kuanyamarum* (Fig. 2(g)) is characterized by the absence of both the first and second groups of peaks but with peaks occurring in the middle region between *m/z* 6800 and 7300.

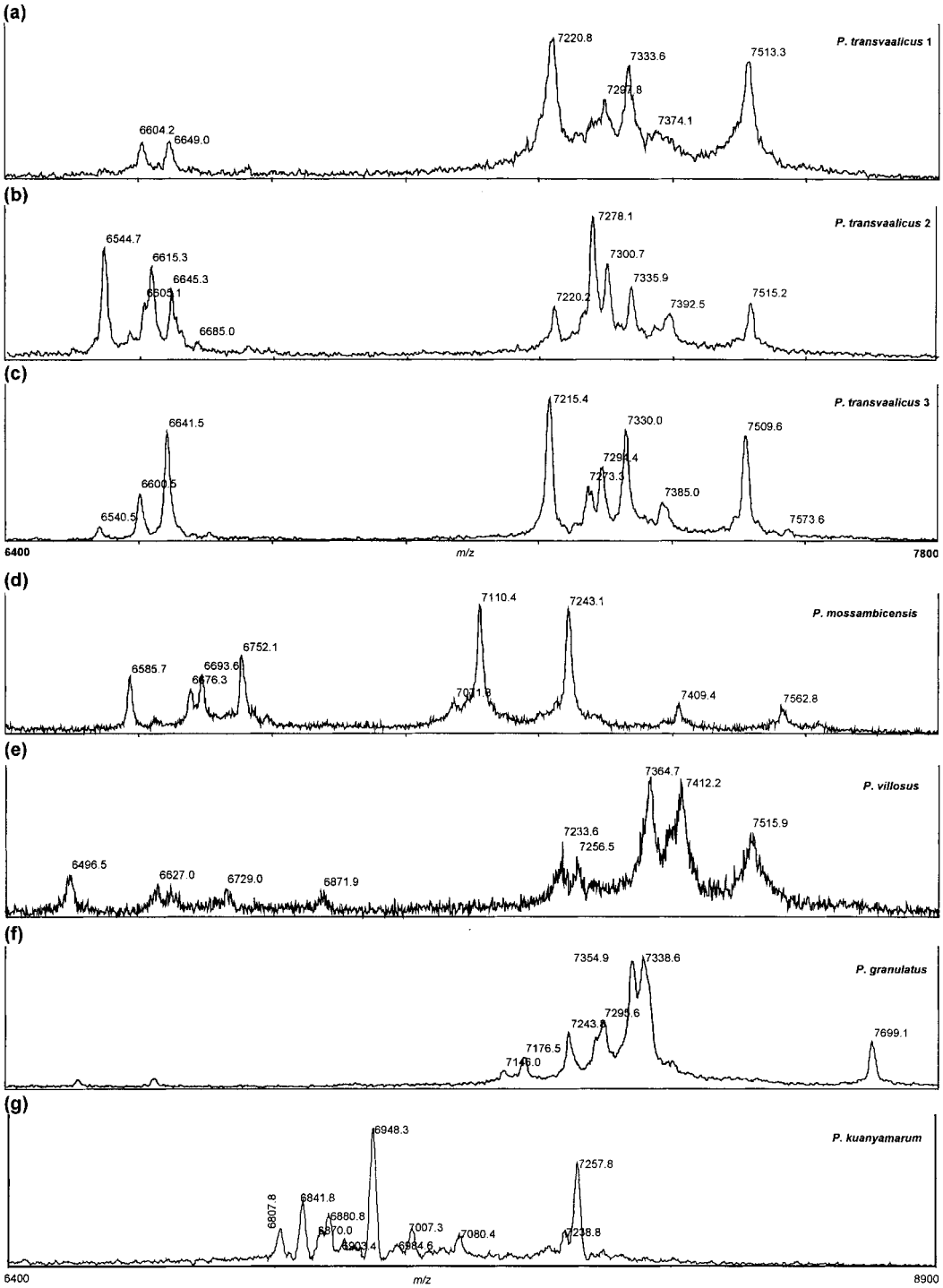

Figure 2. MALDI-TOF mass spectra of (a)–(c) three individuals of *Parabuthus transvaalicus*; (d) *Parabuthus mossambicensis*; (e) *Parabuthus villosus*; (f) *Parabuthus granulatus* and (g) *Parabuthus kuanyamarum* in the mass range *m/z* 6400–7800 (a)–(c) or 8900 (d)–(g) showing resemblances and differences in the patterns of the mass spectra for the different species.

Figure 3 summarizes all the reproducible peaks observed in the selected mass range 6.4–8.4 kDa for 16 representatives of the *Parabuthus* species. From the venom profile it is clear that *P. planicauda* is the only species with peaks only in the first group, *P. granulatus* and *P. kalaharicus* with peaks only in the second group and *P. nanus, P. kuanyamarum* and *P. laevifrons* with peaks in and between the two groups. Peaks in only the first and second groups, comparable to *P. schlechteri* and *P. transvaalicus*, characterize all the other species.

Based on the results presented in Fig. 3, it was possible to construct a venom profile for species identification by selecting only the three most prominent of the reproducible peaks for each species (Table 2). Prominent peaks can be defined as peaks that were always present in the venom and were clearly resolved by MALDI-TOFMS. Unless a parti-

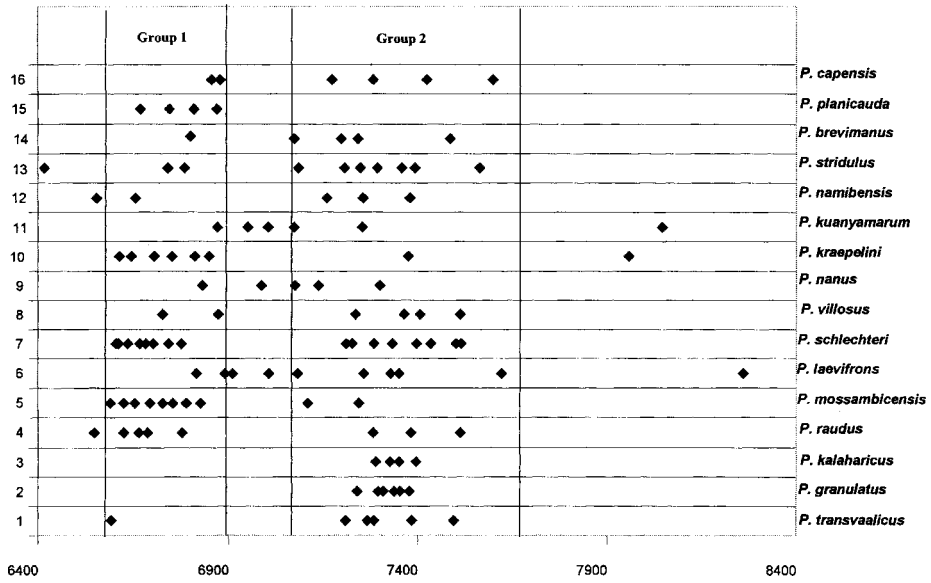

Figure 3. Venom profile of all the reproducible peaks between *m/z* 6400 and 8400 for 16 species of *Parabuthus*.

cular individual scorpion expresses a specific component at an exceptionally low concentration, this three-peptide venom profile allows in most instances direct classification of the species. In order to obtain an estimate of the accuracy of prediction using the *m/z* values in Table 2, a cluster analysis was performed using Ward's method.[41] With equal intervals of 150 Da or smaller, phenograms could be obtained in which the smallest percentage disagreement between any two species was 5.3%. The best result was obtained with intervals of 150 Da (Table 3). A quantitative comparison between phylogeny and venom profiles was not possible using only the mass peaks in the 6.5–8 kDa range. However, the three diagrams obtained using Ward's analysis showed that *P. brevimanus, P. kuanyamarum, P. nanus* and *P. leavifrons* group together, while *P. raudus, P. villosus, P. schlecteri* and *P. transvaalicus* form a distinct group. *P. kalaharicus* and *P. granulatus* showed the smallest percentage disagreement of all the groups indicating that they are closely related. These results correlate with the presently accepted phylogenetic analysis for the species of *Parabuthus*, based on morphology and behaviour.[21]

A species-specific profile may contribute to differentiation between morphologically similar species, especially during various stages of ontogeny when some species are easily confused, e.g. juvenile *P. granulatus* and adult *P. kalaharicus*. These data might also find application in combination with morphological and behavioural data for delimiting species in taxonomically problematic complexes of cryptic species. Until recently, there was some confusion as to whether *P. capensis* and *P. planicauda* were separate species.[42] Some authors thought that *P. capensis* had been redescribed under the name *P. planicauda* and that they were thus conspecific. The matter was resolved only recently by examination of the type material and the two species are now regarded as distinct. Confusion has also beset *P. capensis* and *P. neglectus*. The latter was initially described as separate species from *P. capensis*, with which it was subsequently synonymized, only to be reinstated later on. In the most recent treatment,[42] *P. neglectus* is regarded as synonymous with *P. capensis*. These more recent changes are corroborated by the species-specific

Table 2. Species-specific venom code for 15 species of *Parabuthus*, based on the unique combination of peaks for species identification

Species	*m/z*		
P. transvaalicus	6592	7209	7286
P. kraepelini	6614	6706	7376
P. namibensis	6658	7161	7381
P. raudus	6667	6688	7513
P. schlechteri	6683	7226	7516
P. mossambicensis	6695	6756	7245
P. stridulus	6741	6787	7207
P. planicauda	6747	6811	6871
P. capensis	6856	6879	7287
P. kuanyamarum	6873	7006	7253
P. laevifrons	6911	7259	7328
P. brevimanus	7075	7241	7486
P. granulatus	7241	7338	7358
P. kalaharicus	7328	7351	7395
P. villosus	7364	7408	7512

Table 3. Summary of results obtained from a cluster analysis using Ward's method to determine the smallest percentage disagreement between any two species

Width of interval (Da)	Number of intervals	Smallest percentage (%) disagreement
15	63	6.3
25	38	5.3
50	20	5.0
100	11	9.0
150	7	14.0
200	6	0.0

venom profiles presented here, demonstrating the utility of independent sources of evidence in species delimitation.

CONCLUSIONS

This is the first time to our knowledge that the venoms of medically important scorpions of southern Africa have been systematically examined. MALDI-TOFMS is thus established as a fast, simple and suitable method to identify species-dependent differences in venom composition, and may contribute to fine-tuning of species- and subspecies-dependent characteristics. Such knowledge may be extended to a broader exploration of environmental and nutritional influences on the evolution of venom composition and dynamics of the species. Some of the scorpion species, such as *P. granulatus*, are widely distributed throughout the country and may exhibit geographical variation (polymorphism) in venom composition. However, as only the most reproducible and prominent peaks were used here in determination of the species-specific venom profiles, the peaks that are dependent on the geographical distribution of the species would be eliminated. Although the presence of similar peptides in the venom of different *Parabuthus* scorpions are evident, important dissimilarities occur. This study, performed on a large group of representative *Parabuthus* species, may provide a lead for purifying and exploring specific peptides or searching for specific toxin-related genes.

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