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The complexity and structural diversity of ant venom peptidomes is revealed by mass spectrometry profiling

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Keywords:	Ants, Venom, Peptidome, MALDI-TOF MS, Disulfide bond
Abstract:	<p>RATIONALE: Compared with other animal venoms, ant venoms remain barely explored. Ants have evolved complex venoms to rapidly immobilize arthropod prey and to protect their colonies against predators and pathogens. Many ants have retained peptide-rich venoms similar to other arthropod groups.</p> <p>METHODS: With the goal of conducting a broad and comprehensive survey of ant venom peptide diversity, we have investigated the peptide composition of venoms from 82 stinging ant species, covering 9 subfamilies, using MALDI-TOF mass spectrometry. We also performed an in-depth investigation of eight venoms using reversed-phase (RP)-HPLC separation coupled to offline MALDI-TOF mass spectrometry.</p> <p>RESULTS: Our results reveal that peptide composition of ant venom peptidomes from both poneroid and formicoid ant clades are comprised of hundreds of small peptides (<4 kDa), while large peptides (>4 kDa) are also present in the venom of formicoids. Chemical reduction revealed the presence of disulfide-linked peptides in most ant subfamilies, comprising peptides structured by one, two or three disulfide bonds as well as dimeric peptides reticulated by three disulfide bonds.</p> <p>CONCLUSIONS: The biochemical complexity of ant venoms, associated with an enormous ecological and taxonomic diversity, suggests that stinging ant venoms constitute a promising source of bioactive molecules that could be exploited in the search for novel drug and biopesticide leads.</p>

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**The complexity and structural diversity of ant venom peptidomes is revealed
by mass spectrometry profiling**

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ABSTRACT

RATIONALE: Compared with other animal venoms, ant venoms remain barely explored. Ants have evolved complex venoms to rapidly immobilize arthropod prey and to protect their colonies against predators and pathogens. Many ants have retained peptide-rich venoms similar to other arthropod groups.

METHODS: With the goal of conducting a broad and comprehensive survey of ant venom peptide diversity, we have investigated the peptide composition of venoms from 82 stinging ant species, covering 9 subfamilies, using MALDI-TOF mass spectrometry. We also performed an in-depth investigation of eight venoms using reversed-phase (RP)-HPLC separation coupled to offline MALDI-TOF mass spectrometry.

RESULTS: Our results reveal that peptide composition of ant venom peptidomes from both poneroid and formicoid ant clades are comprised of hundreds of small peptides (<4 kDa), while large peptides (>4 kDa) are also present in the venom of formicoids. Chemical reduction revealed the presence of disulfide-linked peptides in most ant subfamilies, comprising peptides structured by one, two or three disulfide bonds as well as dimeric peptides reticulated by three disulfide bonds.

CONCLUSIONS: The biochemical complexity of ant venoms, associated with an enormous ecological and taxonomic diversity, suggests that stinging ant venoms constitute a promising source of bioactive molecules that could be exploited in the search for novel drug and biopesticide leads.

1. Introduction

Animal venoms are sophisticated biochemical weapons that are currently under intense investigation for their potential to be developed into novel sources, or lead compounds, for therapeutic agents and insect-selective biopesticides. The molecular diversity of venoms, particularly peptide toxins, is linked to a formidable array of molecular targets and pharmacological properties. Several drugs and a biopesticide have already been developed based on the high selectivity and potency of venom peptides and an in-depth exploration of animal venoms will undoubtedly lead to further discoveries [1-3].

Venoms exhibit an extraordinary biochemical complexity ranging from small molecules to large proteins that have been fine-tuned by nature for higher efficacy and target selectivity. In most animal venoms, peptides are the predominant class of toxins and have been investigated intensively in snakes [4], scorpions [5], cone snails [6-7] and spiders [8-10]. However, as the venomous animal biodiversity encompasses ca. 173,000 species, the vast majority of animal venoms remain unexplored in spite of their potential. Several large venomous animal groups have been largely unstudied including centipedes [11], ticks [12], sea anemones [13], wasps [14] and ants. This is largely due to the small size and difficulty of collecting venoms or glands from these invertebrates. However, cutting-edge technologies such as transcriptomic and proteomic approaches now offer the possibility to explore these venoms in detail, with samples of limited size. Many species are also cryptic, difficult to access or can only be identified by specialized taxonomists. In addition, they may not be seen as a potential health threat to humans and therefore have attracted less attention than snakes, scorpions, spiders and cone snails that have been involved in many human fatalities.

Ants (Hymenoptera: Formicidae) are a good example of such a neglected group. Ants are extremely diverse and ubiquitous in terrestrial environments [15] and can be considered as one of the most abundant group of venomous animals on earth. Ants are known to use venoms for both offensive and defensive purposes and are also among the leading predators of other invertebrates in most ecosystems [16]. Some ant subfamilies lack stingers and spray secretions containing formic acid from their venom glands (Formicinae) or deposit small chemicals from their pygidial glands (Dolichoderinae) onto their targets. However, ca. 70% of ant species (ca. 9,000 species) are capable of stinging and inject their venoms via an abdominal apparatus including a stinger connected to a venom gland [17]. Despite this, it is

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2
3 87 fascinating to note that although the total number of stinging ants species is actually higher
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5 88 than the combined number of snake, scorpion and cone snail species, very little is known
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7 89 about their venom composition. Apart from those individuals with an allergy to ant stings,
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9 90 this is largely due to their innocuity and their small size. So far, most work on ant venoms
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11 91 has concentrated on small organic molecules such as hydrocarbons and alkaloids [18-20].
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13 92 However, ant venoms also contain a variety of peptides and proteins [21-22] and therefore
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15 93 are potentially as complex as venoms from other group of arthropods. This variability in the
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17 94 composition of ant venoms may be further enhanced by differing selection pressures due to
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19 95 their enormous ecological diversity [22-23].

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21 96 Recent studies have revealed that stinging ant venoms are rich in peptides, similar to
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23 97 other venomous animals [21]. However, very few ant venom peptides have been
24
25 98 characterised and only 72 peptide sequences have been reported to date. These are mainly
26
27 99 linear peptides lacking disulfide bonds that have antimicrobial, cytolytic and insecticidal
28
29 100 activities. Examples include poneratoxin [24-25], poneritoxins [26], bicarinalins [27] and
30
31 101 dinoponeratoxins [28-29]. Venoms of other arthropods and as well as mollusks and snakes
32
33 102 are known to contain disulfide-rich peptides as their main constituents [9, 30-33]. Although
34
35 103 broad coverage of ant peptides has not been obtained so far, some preliminary data
36
37 104 indicates that ant venoms may also contain various types of peptides cross-linked by
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39 105 disulfide bridges. Disulfide bonds constrain the peptide backbone into rigid three-
40
41 106 dimensional scaffolds which endow the peptides with improved chemical stability, increased
42
43 107 resistance to *in vivo* proteolytic degradation and form tightly defined pharmacophores that
44
45 108 can be finely modulated by single amino acid mutations [34]. These characteristics make
46
47 109 cysteine-rich venom peptides the major active components of animal venoms that are
48
49 110 responsible for their pharmacological activities [9]. In particular, the inhibitor cystine knot
50
51 111 (ICK) structural motif is relatively common in small cysteine-rich peptide toxins from a
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53 112 variety of animal venoms and plants [35-39]. While large numbers of ICK peptide toxins have
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55 113 been reported in other arthropods such as cone snails, sea anemones, spiders and scorpions,
56
57 114 only two such ant venom peptides with a disulfide connectivity consistent with ICK toxins are
58
59 115 currently described – *Dinoponera* ICK-like peptide and SKTXs [40-41]. Indeed, all of the
60
116 disulfide-rich peptides previously isolated from ant venoms were homo- and heterodimeric
117 complexes such as myrmexins, pilosulins, and ectatomins found in venoms from the

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3 118 subfamilies Myrmeciinae [42-43], Pseudomyrmecinae [44] and Ectatomminae [45] (for a
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5 119 complete review see [46]).

6
7 120 In light of the enormous chemical, taxonomical and ecological diversity of ants, we
8
9 121 therefore hypothesized that ant venoms are likely to represent a promising source of unique
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11 122 peptides with original scaffolds and novel pharmacologies. The study of ant venoms may
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13 123 therefore open up a new, and largely unexplored, field in toxinology that may hold great
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15 124 potential in the search for novel drug leads and genetically engineered biopesticides.

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17 125 In earlier studies, we reported a novel method for the investigation of venom peptides
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19 126 and have successfully applied it to the chemotaxonomic study of selected ant species [21].
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21 127 The present study has embarked on a broader investigation of ant venoms, with the aim of
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23 128 conducting a wide survey of ant venom peptidomes among nine different stinging ant
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25 129 subfamilies. The study was designed to provide a comprehensive coverage of ant venom
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27 130 peptide composition, particularly focusing on the discovery of disulfide-linked peptides in
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29 131 these venoms. MS profiling of these ant venoms has revealed the occurrence of hundreds of
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31 132 unknown small linear peptides as well as many novel peptides cross-linked by one, two or
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33 133 three disulfide bonds, suggesting a great depth of structural and probable pharmacological
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35 134 diversity in ant venoms.

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38 136 **2. Materials and methods**

39 137 **2.1. Ant Collection and Taxonomy**

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41 138 Venoms from 82 ant species were investigated in the present study, covering 31 genera
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43 139 from 9 of the 16 stinging ant subfamilies. Field collections of live worker ants were
44
45 140 conducted in various areas of French Guiana, with additional samples sourced from
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47 141 continental France as well as Trinidad and Tobago. In order to include the largest possible
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49 142 coverage of biodiversity and phylogenetic range, additional venoms were purchased from a
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51 143 commercial supplier (Southwest Venoms, Tucson, AZ, USA). The samples included in the
52
53 144 present study represent ca. 1% of the total stinging ant species currently described and ca.
54
55 145 13% of all stinging ant genera (Table 1 and Fig. 1B). Other subfamilies not included in the
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57 146 study are either rare or not present in the collection areas accessible to our group (South-
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59 147 America, Australia and France), such as African ants. Some subfamilies such as the
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148 Amblyoponinae and Cerapachyinae are difficult to investigate due to their subterranean way

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3 149 of life, which makes collection work more prone to serendipity. Also the subfamily
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5 150 Myrmicinae is very large and diversified with ca. 6,500 species [17] and therefore it is
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7 151 difficult to obtain a representative coverage of this subfamily. Phylogenetic analyses were
8
9 152 conducted according to the most recent phylogeny of ants which describes 21 subfamilies
10
11 153 and divides ant subfamilies into 3 clades: leptanilloid, poneroid and formicoid [16, 47]. Ants
12
13 154 belonging to the leptanilloid clade were not included in this study. The complete list of ant
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15 155 species investigated in the present study is provided in Supplementary Tables 1 and 2.

156 Collected ants were stored at -20°C prior to dissection of venom glands and specimens
157 from each of the 82 species were also stored in 96% ethanol for later morphological
158 identification. Three to thirty venom glands from worker ants were dissected for each
159 species, pooled and stored in 10% v/v acetonitrile (ACN) for whole venom analysis. For
160 further exploration by liquid chromatography coupled to mass spectrometry (LC-MS), 8–50
161 venom glands per species were dissected and pooled. Samples were centrifuged for 5 min at
162 14,400 rpm; the supernatant was collected and freeze-dried prior to storage at -20°C .
163 Freeze-dried venoms of *Myrmecia* sp., *Diacamma* sp., *Dinoponera grandis*, *Streblognathus*
164 *aethiopicus*, *Tetraponera* sp. and *Pogonomyrmex maricopa* were purchased from Southwest
165 Venoms.

167 **2.2. Mass spectrometry analysis of crude venoms**

168 MS analyses were performed on a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied
169 Biosystems, CA, USA). Samples were prepared as previously reported using ferulic acid (FA)
170 matrix dissolved in 20% ACN/0.1% v/v trifluoroacetic acid (TFA) at a concentration of 10
171 mg/mL with the addition of 100 mM serine [21]. The FA/serine combination has been
172 previously shown to be the most efficient matrix for the analysis of crude ponerine ant
173 venoms by MALDI-TOF MS [21]. Prior to MS analysis, crude venoms were desalted using
174 ZipTip® C18 pipette tips (Millipore, MA, USA). Then 0.5 μL of desalted sample was deposited
175 on the MALDI target plate followed by 0.5 μL of matrix. Each mass spectrum was calibrated
176 externally using a mixture of peptides of known molecular masses in the same m/z range
177 (Peptide Calibration Mix 4, LaserBio Labs, Sophia-Antipolis, France). 0.5 μL of the calibration
178 mixture was co-crystallized with 0.5 μL of matrix, spotted adjacent to each sample and
179 measured separately. All calibration spectra were acquired in automated mode, to maximize
180 mass accuracy and reproducibility, and were calibrated automatically. Spectra of crude

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3 181 venoms were acquired in linear or reflector mode and calibrated automatically using the
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5 182 sequence module of the Voyager® Control Software (Applied Biosystems, CA, USA). Five
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7 183 spectra of 50 laser shots per spectrum were accumulated for each sample, based on the
8
9 184 acceptance parameters and adequate signal intensity in the 500–10,000 m/z range. Mass
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11 185 spectra were collected in positive ion mode with 20 kHz acceleration. Signals below 500 m/z
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13 186 were not recorded as they were comprised of mostly matrix-related ion clusters [8].
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15 187 16 188 **2.3. RP-HPLC separation**

17 189 In order to obtain a representative picture of ant venom diversity, we selected one venom
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19 190 each from eight different ant subfamilies to be further analysed by LC-MALDI MS. C18
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21 191 reversed-phase high-performance liquid chromatography (RP-HPLC) separation of venoms of
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23 192 the following species was therefore conducted: *Prionopelta* cf. *amabilis* (Amblyoponinae),
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25 193 *Pachycondyla goeldii* (Ponerinae), *Acanthostichus* sp. 1 (Cerapachyinae), *Eciton burchelli*
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27 194 (*Ecitoninae*), *Myrmecia pilosula* (Myrmeciinae), *Tetraoponera* sp. (*Pseudomyrmecinae*),
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29 195 *Gnamptogenys sulcata* (*Ectatomminae*) and *Manica rubida* (*Myrmicinae*). These selected ant
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31 196 venoms were fractionated analysed by using an Xterra-C18 5 μm , 2.1 x 100 mm column
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33 197 (Waters, USA) using a gradient of solvent A (0.1% v/v TFA) and solvent B (ACN/0.1% v/v TFA).
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35 198 The percentage of solvent B was modified as follows: 0% for 5 min, 0–60% over 60 min, 60–
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37 199 90% over 10 min and 90–0% over 15 min, at a flow rate of 0.3 mL/min. The eluate was
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39 200 monitored by UV absorbance at 215 nm on a diode-array detector. All analyses were
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41 201 performed on an HP 1100 HPLC system (Agilent, Santa Clara, CA, USA). Peptide elution was
42
43 202 monitored in real time and fractions were collected manually for each eluting peak. Each
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45 203 fraction was then dried and reconstituted in 50 μL of 0.1% v/v TFA for offline MALDI-TOF MS
46
47 204 analysis and disulfide bond reduction.
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49 205 50 206 **2.4. Disulfide bond reduction and alkylation**

51 207 The presence of disulfide-bonded peptides in ant venoms was determined by chemical
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53 208 reduction of crude venoms and HPLC fractions. 5 μL of crude venom or selected HPLC
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55 209 fractions were incubated with 10 μL of 100 mM ammonium bicarbonate buffer (pH 8)
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57 210 containing 6 M guanidine and 10 mM dithiothreitol (DTT) for 30 min at 56°C. The reaction
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59 211 was stopped by the addition of 5 μL of 0.1% v/v TFA. Prior to MS analysis, reduced venoms
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212 or fractions were desalted using ZipTip® C18 pipette tips (Millipore). Chemical reduction

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3 213 results in a mass increase of 2 Da for each disulfide bond. Thus, by comparing the mass
4 214 spectra of native and reduced samples the number of disulfide bonds in ant venom peptides
5 215 can be determined. However, due to the use of a single-stage MALDI-TOF MS instrument,
6 216 the resolution of the instrument did not permit detection of +2 Da mass differences in
7 217 peptides with masses above 5kDa. Therefore, venom fractions which contained peptide
8 218 masses over 5 kDa were also alkylated, following DTT reduction, by incubating the mixture
9 219 with 50 mM iodoacetic acid (IAA) for 15 min at room temperature in dark. The formation of
10 220 the S-carboxymethyl derivative of cysteine results in a mass increase of 116 Da for each
11 221 disulfide bond (58 Da for each cysteine residue) that allows the determination of the number
12 222 of disulfide bonds in larger venom peptides.
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224 **2.5. Mass spectra analysis**

225 All mass spectra were processed with Data Explorer® 4.11 software (AB SCIEX, Framingham,
226 USA) and subjected to a baseline correction with a correlation factor of 0.7 and Gaussian
227 smoothing to reduce noise with a 5-point filter width. Supplementary masses resulting from
228 sodium (+22 Da) or potassium (+38 Da) adducts were manually removed from all mass lists.
229 Masses matching within ± 1.0 Da in neighbouring HPLC fractions were considered as identical
230 peptides, reflecting incomplete separation, and were also removed. Two-dimensional scatter
231 plots, termed '2D venom landscapes', were constructed using SigmaPlot 12.0 software
232 (Systat, CA, USA). All peptide masses detected in each HPLC fraction were plotted as a
233 function of their m/z values (x -axis) and their HPLC retention time reflecting their
234 hydrophobicity (y -axis). Averaged data represents the mean \pm S.D., unless otherwise stated.
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236 **3. Results**

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238 **3.2. Crude venom analysis**

239 Crude venoms of the 82 ant species were initially analyzed by MALDI-TOF mass spectrometry
240 and a total of 1396 distinct masses were detected in all venoms after elimination of masses
241 consistent with doubly charged ions, or potassium and sodium adducts. The mean number
242 of peptides detected in crude venom profiles was 17 ± 9 and varied widely, ranging from 4 to
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3 243 42 in *Chalepoxenus muellerianus* (Myrmicinae) and *Solenopsis saevissima* (Myrmicinae)
4
5 244 venoms, respectively.

6 245 The analysis of crude venoms revealed a great heterogeneity in the masses of peptides
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8 246 between subfamilies with an overall mass range of 652.2–8569.4 m/z . The huge variation of
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10 247 peptide composition between subfamilies is illustrated by the narrow mass range of
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12 248 peptides of Amblyoponinae (2138.8–3782.6 m/z) as opposed to the broad mass range of
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14 249 peptides in both Ponerinae and Ecitoninae subfamilies (685.7–6992.0 m/z and 1019.5–
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16 250 8569.4 m/z , respectively). Mass analysis of all crude venoms revealed the presence of mostly
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18 251 small peptides with 87% of all masses below 4 kDa (Fig. 1A). However, larger peptides with
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20 252 masses >4 kDa were also observed in specific venoms, notably in subfamilies belonging to
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22 253 the formicoid clade, where $22.9 \pm 23.7\%$ ($n = 41$ species investigated) of peptides have
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24 254 masses >4 kDa. The proportion of large peptides in ant venoms is even greater in Ecitoninae
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26 255 venoms with $56.3 \pm 24.3\%$ ($n = 8$ species investigated) of peptides in the mass range 4000.8–
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28 256 8569.4 m/z . This finding is consistent with previous reports of large peptides described and
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30 257 sequenced from formicoid ant venoms such as ectatomins [48], myrmexins [44] and
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32 258 pilosulins [42-43, 49]. It should be noted that the larger peptide masses are associated with
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34 259 dimeric forms of peptides, often associating two linear chains linked by interchain disulfide
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36 260 bonds [46]. In the range of species studied, the proportion of large peptides (>4 kDa) found
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38 261 in ant venoms from the poneroid clade was significantly lower than in the formicoid clade
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40 262 (Mann-Whitney $U = 280$, $n_1 = n_2 = 41$, $P < 0.001$). Only $3.0 \pm 6.1\%$ ($n = 39$ species investigated)
41
42 263 of peptides of ponerine ants were found to have masses >4 kDa (mass range 4049.8–6992.0
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44 264 m/z). This result confirms our earlier observations of Ponerinae ant venom composition,
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46 265 particularly from the *Pachycondyla* and *Odontomachus* genera, where 99% of all masses
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48 266 detected were in the mass range 0.5–5.0 kDa[21]. However, despite the narrow range of
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50 267 masses, there are significant differences between genera and between species belonging to
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52 268 the same genus as illustrated by results from ten different genera in the subfamily Ponerinae
53
54 269 (Fig. 1C) and species from the broad genus *Pachycondyla* (Fig. 1D).

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56 270 Overall, examination of crude ant venom peptidomes indicates that ant venoms may
57
58 271 contain primarily small peptides in the mass range 0.5–4.0 kDa with larger peptides
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60 272 observed mostly in the formicoid clade. We can therefore estimate that the theoretical sizes
273 of most ant venom peptides are below 35 residues. This estimate was determined from a
274 MW_{av} of 111.1254 Da using the statistical occurrences of amino acids in proteins, of an

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3 275 average amino acid, averagine, calculated with the formula $C_{4.9384}H_{7.7583}N_{1.3577}O_{1.4773}S_{0.0417}$
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5 276 [50]. This finding is similar to conotoxins that are typically between 10 and 30 amino acids in
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7 277 length [51] but is in contrast to spider, scorpion and snake venom peptide toxins that are
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9 278 normally between 40 and 100 amino acids [52].
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280 **3.3. Disulfide bond mapping in crude venoms**

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13 281 To broadly map the presence of disulfide-bonded peptides in crude venoms, venoms were
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15 282 reduced and comparative MALDI-TOF MS profiling of native venoms revealed the presence
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17 283 of ca. 50 peptides linked by disulfide bonds. We detected disulfide-bonded peptides among
18
19 284 five distinct ant subfamilies from both formicoid and poneroid clades (Ponerinae,
20
21 285 Amblyoponinae, Ectatomminae, Myrmeciinae and Myrmicinae). Comparison of the spectra
22
23 286 before, and after, reduction permitted us to detect the presence of peptides with one, two
24
25 287 or three disulfide bonds (Fig. 2A-C). These masses are listed in Table 2.

26
27 288 Only 32 peptides with 1 disulfide bond were detected in 14 crude venoms
28
29 289 (*Pachycondyla commutata*, *P. mesonotalis*, *P. unidentata*, *Odontomachus hastatus*, *O.*
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31 290 *scalptus*, *Anochetus horridus*, *A. cf. diegensis*, *Streblognathus aethiopicus*, *Prionopelta cf.*
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33 291 *amabilis*, *Ectatomma brunneum*, *Myrmecia rufinodis*, *M. similima*, *Pogonomyrmex*
34
35 292 *maricopa*, *Manica rubida*); 17 peptides with 2 disulfide bonds were detected only in venoms
36
37 293 from the genus *Anochetus* (*A. horridus*, *A. cf. diegensis* and *A. emarginatus*); and 1 peptide
38
39 294 with 3 disulfide bonds was detected in *P. unidentata* venom. From our sample survey, more
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41 295 disulfide-linked peptides were detected in venoms from the poneroid clade (42 disulfide-
42
43 296 bonded peptides) than in the formicoid clade (8 disulfide-bonded peptides). Overall, 33
44
45 297 disulfide-bonded peptides were found in Ponerinae venoms plus 9 in Amblyoponinae
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47 298 venoms (all poneroids), whereas only 1 disulfide-bonded peptide was found in
48
49 299 Ectatomminae, 2 in Myrmeciinae and 5 in Myrmicinae venoms (from the formicoid clade).
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51 300 This may indicate greater structural diversity in the Ponerinae, although a larger venom
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53 301 sample set encompassing more species would be needed for any definitive conclusion to be
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55 302 drawn.

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57 303 While disulfide-bonded peptides seem to be minor components in most ant venoms,
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59 304 some venoms appeared to deviate from this pattern and were composed mostly of disulfide-
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305 bonded peptides. 54% of the 37 peptides detected in venoms of the 3 *Anochetus* species
306 (Ponerinae) were structured by 1 and 2 disulfide bonds and 90% of the 10 peptides detected

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3 307 in venom of *Prionopelta cf. amabilis* (Amblyoponinae) were structured by 1 disulfide bond.
4 308 This suggests that our study, although sampling a broad range of ant subfamilies, cannot
5 309 reveal the full extent of ant peptidome diversity. The vast number of stinging ant species (ca.
6 310 9,000 species) may reveal other peptide classes, structures and pharmacological properties.
7 311 This is because specific genera like *Anochetus* and *Prionopelta* may have evolved different
8 312 venoms based on atypical structural motifs.
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14 314 **3.4. LC-MALDI analysis and 2D landscapes**

15 315 Mass fingerprinting of crude venoms revealed up to a maximum of only 42 peptides, as seen
16 316 with *Solenopsis saevissima* venom. However, this low peptide count is consistent with
17 317 results obtained from a series of tarantula venoms where examination of crude venoms by
18 318 MALDI-TOF MS revealed a maximum of 50 to 70 peptides [53-56]. Further examination of
19 319 HPLC fractions later revealed the full complexity of these tarantula venoms [8]. Crude venom
20 320 mass fingerprinting is therefore a suitable tool for preliminary investigation and for
21 321 comparing venom profiles in a given taxonomic group. However, it cannot reveal the full
22 322 extent of the venom peptidome, most likely due to ion suppression effects where large
23 323 numbers of peptides compete for proton capture during the MALDI ionization process.
24 324 Consequently, the most abundant peptides, or the ones with the highest proton affinity, are
25 325 likely to be over-represented in the mass spectra, while peptides with low abundance or
26 326 lesser proton affinity may not ionize, or ionize in amounts below the detection limit. This
27 327 results in spectra displaying only a subset of the crude venom complexity. The key to
28 328 revealing the full extent of a venom peptidome diversity is chromatographic separation of
29 329 the venom prior to mass analysis, using hyphenated chromatography either online (LC
30 330 coupled to electrospray ionization MS) or offline (LC coupled to sample deposition and
31 331 MALDI-TOF MS). We therefore proceeded to fractionate a range of ant venoms into fractions
32 332 containing less peptides to reveal the true venom complexity.

33 333 The resultant chromatographic profiles showed few similarities between the various
34 334 subfamilies (Fig. 3) and varied in their complexity. This is reflected by the number of
35 335 fractions collected from the chromatograms. A total of 27 fractions were collected from the
36 336 least complex venom (*Tetraponera* sp.) and 61 from the most complex venom (*Eciton*
37 337 *burchelli*). The overall complexity and range of elution patterns observed in ant venoms is
38 338 parallel to that observed with RP-HPLC profiles of other venomous arthropods such as
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3 339 Australian funnel-web spiders [10]. The eight ant venoms that were fractionated showed
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5 340 peptides eluting in a broad range of hydrophobicity. The most hydrophilic fraction eluted at
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7 341 10.8% ACN (Fraction *f*1 from the venom of *Eciton burchelli* at 15.8 min) and the most
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9 342 hydrophobic fraction eluted at 60% ACN (*f*43 from the venom of *Pachycondyla goeldii* at 65
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11 343 min). Venom of *Prionopelta cf. amabilis* eluted over a narrow hydrophobicity range (15.9–
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13 344 35.2% ACN) while both *Manica rubida* and *Pachycondyla goeldii* venoms eluted in a broad
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15 345 hydrophobicity range (13.1–57% ACN and 15–60% ACN, respectively). This highlights the
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17 346 broad physicochemical diversity among ant venom peptides, suggesting that peptides with
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19 347 different structural scaffolds or widely differing amino acid compositions are present.

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21 348 Chromatographic fractions were then analysed by offline MALDI-TOF MS leading to the
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23 349 construction of bi-dimensional graphs termed '2D venom landscapes' in which the molecular
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25 350 mass of each peptide in a given venom was plotted against HPLC retention time reflecting
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27 351 hydrophobicity. The 2D landscapes revealed the enormous peptide complexity in ant
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29 352 venoms (Fig. 4). The total number of masses detected in the venoms analysed varied from
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31 353 31 to 288 for *Prionopelta cf. amabilis* and *Pachycondyla goeldii*, respectively (Fig. 5). Some
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33 354 fractions were highly complex and although they were collected as single peaks based on
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35 355 signal monitoring, mass analysis revealed the presence of up to 26 peptides in a single
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37 356 fraction (*f*18 at a retention time 32.6 min from *Pachycondyla goeldii*). This clearly
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39 357 demonstrates the power of the 2D analytical approach, as true venom complexity is not
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41 358 revealed by single-dimensional analysis.

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43 359 Although many more peptides were detected, the mass distribution of peptides from
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45 360 the LC-MALDI-TOF MS analyses was quite similar to those previously observed in the analysis
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47 361 of the corresponding crude venoms. A total number of 1112 peptides were detected within
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49 362 the 8 venoms by LC-MALDI-TOF MS and 94.4% of all peptides detected by LC-MALDI-TOF MS
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51 363 have a mass less 5 kDa and 87.5% below 4 kDa. However, peptides in the venoms of some
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53 364 formicoids have a broad mass range such as *Eciton burchelli* (592.2–7595.0 *m/z*), *Myrmecia*
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55 365 *pilosula* (679.5–6246.4 *m/z*), *Tetraponera* sp. (1012.2–5773.2 *m/z*) and *Manica rubida*
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57 366 (535.4–6570.0 *m/z*), with many peptides of masses >4 kDa, while the other formicoid and
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59 367 poneroid venoms contain peptides that were almost exclusively less than 4 kDa (Fig.4). In
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368 contrast, some venoms were mostly composed of peptides in a remarkably narrow mass
369 range. This included venoms such as *Prionopelta cf. amabilis* that contained only 31 peptides
370 with masses ranging from 1411.1 to 4265.2 *m/z* (Fig. 5).

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372 **3.5. Disulfide bond mapping**

373 For a more precise characterization of disulfide-bonded peptides in each venom, chemical
374 reduction of each HPLC fraction was carried out to map the presence and number of
375 disulfide bonds. We detected the presence of monomeric peptides with one, two or three
376 disulfide bonds within all the venoms tested (Fig. 4). Peptides reticulated by one disulfide
377 bond were found in a wide range of venoms including *Prionopelta* cf. *amabilis*, *Pachycondyla*
378 *goeldii*, *Tetraoponera* sp., *Myrmecia pilosula*, *Gnamptogenys sulcata* and *Manica rubida*. The
379 masses of these peptides ranged from 1274.2 *m/z* (*Pachycondyla goeldii*) to 4701.8 *m/z*
380 (*Tetraoponera* sp.). Peptides with 2 disulfide bonds were identified in the venom of *Eciton*
381 *burchelli* within the mass range 6594–7595 *m/z* (Fig. 4D). Peptides with two disulfide bonds
382 were also identified in the crude venom analysis of *Anochetus emarginatus*, *A. cf. diegensis*
383 and *A. horridus* venoms. However, the mass of these peptides was much lower compared to
384 those of *Eciton burchelli* (1623.6–2709.1 *m/z*), as shown in Table 2. This difference in mass
385 ranges may indicate that these peptides belong to distinct toxin families. A group of 15 linear
386 ponerocins were previously characterized from the venom of *Pachycondyla goeldii* [26]. In
387 the present study, 11 of these ponerocins (G1, G2, G3, G4, G5, G6, L1, L2, W3 and W5) were
388 also detected within the 2D venom landscape of *P. goeldii* (Fig. 4B).

389 Disulfide bond mapping of HPLC fractions revealed peptides with 3 disulfide bonds in
390 the venoms of *Pachycondyla goeldii*, *Acanthostichus* sp. 1, *Eciton burchelli* and *Manica*
391 *rubida* within the broad mass range of 3651.2–6440.8 *m/z*. Both homo- and heterodimeric
392 peptides (Tsp-ab and Tsp-bb) were also found in the venom of *Tetraoponera* sp.
393 (*Pseudomyrmecinae*) with a mass shift, after reduction and alkylation, consistent with the
394 presence of three disulfide bonds (Fig. 2D). It is presently unclear, however, if each Tsp
395 monomer is linked to its other subunit by three inter-chain disulfide bonds or each Tsp
396 monomer has only one inter-chain disulfide and an additional intra-chain disulfide bond.
397 Myrmexins are dimeric peptides that have been previously reported in the venoms of
398 *Pseudomyrmex triplarinus* and *P. penetrator* (*Pseudomyrmecinae*) [44, 57]. However,
399 myrmexin peptides only have two cysteine residues per monomer not three. Although the
400 linear pilosulin 1 (and its isoforms [Ile⁵]pilosulin 1, pilosulin 1 65→112 and pilosulin 1
401 68→112) and the heterodimeric pilosulin 3 were found within the venom of *Myrmecia*
402 *pilosula* (Fig. 4E), the homodimeric pilosulins 4 and 5 were not detected, even though these

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3 403 peptides have been previously reported in *M. pilosula* venom [42-43]. Overall, the
4 404 percentage of disulfide-linked peptides in venoms ranges from 3 to 20% for *M. pilosula* and
5 405 *Tetraponera* sp., respectively (Fig. 5), highlighted by the dominance of linear peptides in ant
6 406 venoms. However, the venom of *Prionopelta* cf. *amabilis* stood out with 32.3% of the 31
7 407 peptides having 1 disulfide bond (Fig. 5).
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17 411 4. Discussion

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20 413 Ant venoms evolved bioactive peptides in order to disrupt multiple biological targets and
21 414 permit the capture of arthropod prey, deter predators, communicate with other members of
22 415 the colony and also act as antimicrobials. It is therefore not surprising that the present study
23 416 of the ant venom peptidome has revealed enormous variation in the complexity of ant
24 417 venoms highlighting the potentially diverse pharmacologies and functions of ant venom
25 418 peptides. It is now well established from other taxonomic groups [7-8] that venoms are
26 419 highly complex, containing up to 1000 peptides, and that many peptide classes as well as
27 420 numerous isoforms in each class combine into a complex biochemical cocktail. Our
28 421 investigation of ant venoms reveals that a wide range of ants produce venoms with a
29 422 peptidic composition as complex as those of spiders, scorpions or cone snails as previously
30 423 demonstrated in the venom of the ponerine ant *Dinoponera quadricaps* [28]. LC-MALDI-TOF
31 424 mass fingerprinting of a wide range of poneroid and formicoid ant venoms detected total
32 425 numbers of peptides ranging from 31 to 288 peptides. Indeed, venoms of *Pachycondyla*
33 426 (*Ponerinae*) ants seem to be particularly rich in peptide components and the total number of
34 427 peptides appear to be as complex as the previously described venom from the related
35 428 ponerine ant *Dinoponera quadricaps* [28]. Based on the present study, we would estimate
36 429 that ant venoms contain on average ca. 130 unique peptides. If we assume a total of ca.
37 430 9,000 stinging ant species, we can calculate the total number to be more than 1 million
38 431 peptides in all stinging ant venoms. However, this number is probably an under-estimate, as
39 432 this does not take into account intra-species variations observed in ant venoms [28].
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56 433 Moreover, cryptic ant species also contribute to a hidden peptide diversity as the venom
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3 434 peptide profile can vary among cryptic ant species [21]. Thus, many ant species are still
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5 435 undiscovered particularly within tropical areas and the total number of ants could reach
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7 436 25,000 species (currently ca. 13,000 described species) [58].

8 437 Hymenopteran venom peptides have not been extensively investigated to date.
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10 438 However, past studies on wasp venoms revealed small linear peptides [14, 59-61] and some
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12 439 rare single disulfide-linked peptides such as pallipine-I, -II and -III from the venom of the
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14 440 wasp *Agelaia p. pallipes* [14] and sylverin from the venom of *Protonectarina sylveirae* [62]. A
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16 441 wide survey of ant venom peptidomes has never been attempted before and this study
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18 442 therefore constitutes the most extensive overview conducted to date, providing a broad
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20 443 overview of the variability of ant venom peptide composition. Ant venom peptides appear to
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22 444 be similar in mass to those of wasps as the majority of their peptides were also less than 4
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24 445 kDa and were devoid of disulfide bonds. We have also shown that formicoid ants still
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26 446 possess some large peptides, which is consistent with earlier studies on ant venoms that
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28 447 showed peptides larger than 4 kDa among the formicoid subfamilies Ectatomminae,
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30 448 Pseudomyrmecinae and Myrmeciinae [42-45, 63]. Given their impressive ecological diversity,
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32 449 formicoid ants may therefore have enhanced their venom arsenal by the development of
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34 450 more complex and larger peptides that permit them to modulate novel pharmacological
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36 451 targets.

37 452 Disulfide-bonded peptides often act on ion channels and receptor targets and are
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39 453 common and dominant components in the venoms of a wide range of evolutionarily
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41 454 unrelated predators including sea anemones, cone snails, centipedes, scorpions, spiders and
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43 455 snakes [11, 33]. The present study has highlighted the distribution of monomeric peptides
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45 456 structured by one, two or three disulfide bonds in a wide range of ant venom peptidomes.
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47 457 From the present study, such peptides appear to be minor components within most ant
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49 458 venom peptidomes except for some venoms such as *Anochetus emarginatus*, *A. cf.*
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51 459 *diegensis*, *A. horridus* and *Prionopelta cf. amabilis* that are mostly composed of disulfide-
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53 460 linked peptides. Peptides structured by one disulfide bond have only been previously
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55 461 identified in the venoms of *Pseudomyrmex gracilis* and *P. penetrator* [57]. In the present
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57 462 study, peptides containing a single disulfide bond were extended to include venoms from 5
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59 463 additional ant subfamilies encompassing 14 ant species (Table 2). In hymenopteran venoms,
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464 monomeric peptides reticulated by two disulfide bonds, such as apamin, were only
465 described from the defensive venom of the honey bee *Apis mellifera* [64]. Importantly, the

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3 466 presence of 17 such peptides from 3 species of *Anochetus* is the first report of peptides with
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5 467 2 disulfide bonds in ant venoms.

6 468 The *Dinoponera* ICK-like peptide [41] is the only monomeric peptide structured by more
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8 469 than one disulfide bond previously described from ant venoms. Peptides sharing high
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10 470 homology with an ICK peptide from tarantula venom have been found in the venom of the
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12 471 myrmecine ant *Strumigenys kumadori* [40], however this data remains unpublished.
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14 472 Importantly, we found 1 peptide in the crude venom of *Pachycondyla unidentata* and several
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16 473 peptides from the venom of *P. goeldii* reticulated by 3 disulfide bonds that had masses
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18 474 within the most common peptide mass range of other ICK toxins (3500–4500 Da). Therefore,
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20 475 this strongly suggests that this toxin scaffold, which has evolved many times in unrelated
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22 476 venomous animals [65], may also be present in many other ant venoms. However, the
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24 477 definitive identification of an ICK structural motif will require purification of the peptides and
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26 478 determination of disulfide linkage and tertiary structure. All the remaining disulfide-bonded
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28 479 peptides identified in ant venoms were homo- or heterodimeric complexes formed from
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30 480 monomers linked by one or two disulfide bonds from *Pseudomyrmex* spp.
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32 481 (*Pseudomyrmecinae*) and *Ectatomma* spp. (*Ectatomminae*) or *Myrmecia pilosula*
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34 482 (*Myrmeciinae*) [23, 44-45, 57, 66]. We have also found dimeric peptides within the venom of
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36 483 the related pseudomyrmecinae ant *Tetraoponera* sp.

37 484 Except for the heterodimeric peptide pilosulin 3 (5603 m/z), no other dimeric peptides
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39 485 were identified in either *Myrmecia pilosula*, *Ectatomma tuberculatum* or *E. brunneum*
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41 486 venoms, even though several different dimeric pilosulins (8192-8540 m/z) and ectatomins
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43 487 (7923-9419 m/z) peptides have been fully characterised from these venoms [42-43]. The
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45 488 ability of our MALDI-TOF MS method using FA/serine matrix to mainly detect peptides in the
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47 489 mass range ca. 500–8000 m/z may explain why the dimeric ectatomins and pilosulins were
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49 490 not detected in our investigation. This shows that the peptide richness is likely to be even
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51 491 more extensive. The use of complementary analyses, employing other techniques such as ESI
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53 492 mass spectrometry or using different matrices, may reveal the presence of larger peptides.

54 493 The present work constitutes the most extensive study of ant venom peptidomes and
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56 494 demonstrates the diversity in mass and disulfide connectivity of peptides in ant subfamilies
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58 495 and species. Ant venoms remain barely investigated and the impressive diversity of peptides
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60 496 from ant venoms highlighted in this work combined with the improvement of mass
497 spectrometry technology should drive future studies on ant venom toxins. Furthermore, the

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3 498 small sizes of ant venom peptides make sequencing and synthesis of these toxins relatively
4 499 facile therefore facilitating characterisation of their pharmacological targets. Ant venoms
5 500 may therefore provide a novel source of bioactive peptides to develop drug and
6 501 bioinsecticide lead compounds.
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Table 1. Genera and extant species of stinging ants

Clade	Subfamily	No. of extant genera	No. of genera studied	% of studied genera	No. of extant species	No. of species studied	% of studied species
poneroid	Amblyoponinae	13	1		121	1	
	Paraponerinae	1	1		1	1	
	Ponerinae	28	10		1157	39	
	Martialinae	1	0		1	0	
	Proceratinae	3	0		135	0	
	Agroecomyrmecinae	2	0		3	0	
formicoid	Cerapachyinae	7	1		267	2	
	Ecitoninae	5	4		151	8	
	Myrmeciinae	2	1		92	4	
	Pseudomyrmecinae	3	2		229	4	
	Ectatomminae	4	2		270	10	
	Myrmicinae	142	9		6443	13	
	Leptanilloidinae	3	0		15	0	
	Aenictinae	1	0		174	0	
leptanilloid	Heteroponerinae	3	0		24	0	
	Leptanillinae	6	0		59	0	
Total		224	31	13.84%	9142	82	0.90%

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Table 2. List of monomeric disulfide bonded peptides detected in crude ant venoms. Data shows presence of one, two and three disulfide bonds detected by shifting of peptide masses (+ 2 Da for each disulfide bond) after reduction with DTT.

No of S-S	MW oxidised [M+H ⁺] (Da)	MW reduced [M+H ⁺] (Da)	Species	Subfamily	Clade
1	2243.34	2245.36	<i>Pachycondyla commutata</i>		
	3059.45	3061.56	<i>Pachycondyla mesonotalis</i>		
	2582.36	2584.36	<i>Pachycondyla unidentata</i>		
	1777.74	1779.70	<i>Odontomachus hastatus</i>		
	2098.87	2100.92	<i>Odontomachus hastatus</i>		
	2282.86	2284.95	<i>Odontomachus hastatus</i>		
	3543.46	3545.62	<i>Odontomachus hastatus</i>		
	2000.74	2002.72	<i>Odontomachus scalptus</i>	Ponerinae	
	2058.75	2060.72	<i>Odontomachus scalptus</i>		
	1459.65	1461.52	<i>Anochetus horridus</i>		
	1702.70	1704.67	<i>Anochetus horridus</i>		
	2283.06	2285.11	<i>Anochetus horridus</i>		poneroid
	1177.59	1179.43	<i>Anochetus cf. diegensis</i>		
	1493.76	1495.64	<i>Anochetus cf. diegensis</i>		
	1633.93	1635.91	<i>Streblognathus aethiopicus</i>		
	2138.83	2140.86	<i>Prionopelta cf. amabilis</i>		
	2154.82	2156.85	<i>Prionopelta cf. amabilis</i>		
	2403.77	2405.80	<i>Prionopelta cf. amabilis</i>		
	2658.20	2660.97	<i>Prionopelta cf. amabilis</i>		
	2665.87	2667.92	<i>Prionopelta cf. amabilis</i>	Amblyoponinae	
2766.80	2768.91	<i>Prionopelta cf. amabilis</i>			
2800.98	2802.97	<i>Prionopelta cf. amabilis</i>			
2820.93	2823.02	<i>Prionopelta cf. amabilis</i>			
2922.90	2925.00	<i>Prionopelta cf. amabilis</i>			
1803.57	1805.52	<i>Ectatomma brunneum</i>	Ectatomminae		
1391.56	1393.66	<i>Myrmecia rufinodis</i>	Myrmeciinae		
1494.87	1496.76	<i>Myrmecia simillima</i>			
1538.71	1540.62	<i>Pogonomyrmex maricopa</i>		formicoid	
2052.93	2054.95	<i>Pogonomyrmex maricopa</i>			
2489.00	2491.12	<i>Pogonomyrmex maricopa</i>	Myrmicinae		
1435.85	1437.89	<i>Manica rubida</i>			
2745.30	2747.26	<i>Manica rubida</i>			
2	1687.70	1691.71	<i>Anochetus horridus</i>		
	1832.80	1836.80	<i>Anochetus horridus</i>		
	2043.85	2047.89	<i>Anochetus horridus</i>		
	2709.10	2713.20	<i>Anochetus horridus</i>		
	1683.67	1687.58	<i>Anochetus cf. diegensis</i>		
	1702.79	1706.71	<i>Anochetus cf. diegensis</i>		
	1958.88	1962.80	<i>Anochetus cf. diegensis</i>		
	2134.88	2138.80	<i>Anochetus cf. diegensis</i>		
	1623.60	1627.59	<i>Anochetus emarginatus</i>	Ponerinae	poneroid
	1716.65	1720.59	<i>Anochetus emarginatus</i>		
	1849.74	1853.74	<i>Anochetus emarginatus</i>		
	1866.76	1870.70	<i>Anochetus emarginatus</i>		
	1916.79	1920.71	<i>Anochetus emarginatus</i>		
	1975.71	1979.69	<i>Anochetus emarginatus</i>		
	2026.79	2030.77	<i>Anochetus emarginatus</i>		
2057.87	2061.82	<i>Anochetus emarginatus</i>			
2093.82	2097.80	<i>Anochetus emarginatus</i>			
3	4434.29	4440.30	<i>Pachycondyla unidentata</i>	Ponerinae	poneroid

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Supplementary Table 1. List of poneroid ants and their origin

Subfamily	Genus	Species	Origin
Amblyoponinae	<i>Prionopelta</i>	<i>cf. amabilis</i>	French Guiana
Paraponerinae	<i>Paraponera</i>	<i>clavata</i>	French Guiana
	<i>Pachycondyla</i>	<i>goeldii</i>	French Guiana
	<i>Pachycondyla</i>	<i>stigma</i>	French Guiana
	<i>Pachycondyla</i>	<i>commutata</i>	French Guiana
	<i>Pachycondyla</i>	<i>villosa</i>	French Guiana
	<i>Pachycondyla</i>	<i>crassinoda</i>	French Guiana
	<i>Pachycondyla</i>	<i>procidua</i>	French Guiana
	<i>Pachycondyla</i>	<i>constricta</i>	French Guiana
	<i>Pachycondyla</i>	<i>harpax</i>	French Guiana
	<i>Pachycondyla</i>	<i>apicalis</i>	French Guiana
	<i>Pachycondyla</i>	<i>unidentata</i>	French Guiana
	<i>Pachycondyla</i>	<i>arhuca</i>	French Guiana
	<i>Pachycondyla</i>	<i>marginata</i>	French Guiana
	<i>Pachycondyla</i>	<i>inversa</i>	French Guiana
	<i>Pachycondyla</i>	<i>mesonotalis</i>	French Guiana
	<i>Pachycondyla</i>	<i>verenae</i>	French Guiana
	<i>Pachycondyla</i>	sp.	French Guiana
	<i>Odontomachus</i>	<i>hastatus</i>	French Guiana
	<i>Odontomachus</i>	<i>haematodus</i>	French Guiana
	<i>Odontomachus</i>	<i>scalptus</i>	French Guiana
Ponerinae	<i>Odontomachus</i>	<i>biumbonatus</i>	French Guiana
	<i>Odontomachus</i>	<i>mayi</i>	French Guiana
	<i>Odontomachus</i>	<i>cf. ruginodis</i>	French Guiana
	<i>Anochetus</i>	<i>emarginatus</i>	French Guiana
	<i>Anochetus</i>	<i>cf. diegensis</i>	French Guiana
	<i>Anochetus</i>	<i>horridus</i>	French Guiana
	<i>Centromyrmex</i>	sp.	French Guiana
	<i>Leptogenys</i>	<i>unistimulosa</i>	French Guiana
	<i>Leptogenys</i>	sp. 1	French Guiana
	<i>Leptogenys</i>	sp. 2	French Guiana
	<i>Leptogenys</i>	sp. 3	French Guiana
	<i>Platythyrea</i>	sp. 1	French Guiana
	<i>Platythyrea</i>	sp. 2	French Guiana
	<i>Platythyrea</i>	sp. 3	French Guiana
	<i>Hypoponera</i>	sp. 1	French Guiana
	<i>Hypoponera</i>	sp. 2	French Guiana
	<i>Hypoponera</i>	sp. 3	French Guiana
	<i>Diacamma</i>	sp.	Unknown
	<i>Dinoponera</i>	<i>grandis</i>	Brazil
	<i>Streblognathus</i>	<i>aethiopicus</i>	Unknown

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Supplementary Table 2. List of formicoid ants and their origin

Subfamily	Genus	Species	Origin
Cerapachyinae	<i>Acanthostichus</i>	sp. 1	French Guiana
	<i>Acanthostichus</i>	sp. 2	French Guiana
Ecitoninae	<i>Eciton</i>	<i>burchelli</i>	French Guiana
	<i>Eciton</i>	<i>mexicanum</i>	French Guiana
	<i>Neivamyrmex</i>	sp. 1	French Guiana
	<i>Neivamyrmex</i>	sp. 2	French Guiana
	<i>Neivamyrmex</i>	sp. 3	French Guiana
	<i>Neivamyrmex</i>	sp. 4	French Guiana
	<i>Labidus</i>	<i>coecus</i>	French Guiana
Ectatomminae	<i>Nomamyrmex</i>	<i>hartigi</i>	French Guiana
	<i>Ectatomma</i>	<i>tuberculatum</i>	French Guiana
	<i>Ectatomma</i>	<i>brunneum</i>	French Guiana
	<i>Ectatomma</i>	<i>edentatum</i>	French Guiana
	<i>Ectatomma</i>	cf. <i>ruidum</i>	Trinidad and Tobago
	<i>Gnamptogenys</i>	<i>sulcata</i>	French Guiana
	<i>Gnamptogenys</i>	<i>striatula</i>	French Guiana
	<i>Gnamptogenys</i>	<i>triangularis</i>	French Guiana
	<i>Gnamptogenys</i>	<i>mordax</i>	French Guiana
	<i>Gnamptogenys</i>	sp. 1	French Guiana
Myrmeciinae	<i>Gnamptogenys</i>	sp. 2	French Guiana
	<i>Myrmecia</i>	<i>gulosa</i>	Australia
	<i>Myrmecia</i>	<i>rufinodis</i>	Australia
	<i>Myrmecia</i>	<i>simillima</i>	Australia
Myrmicinae	<i>Myrmecia</i>	<i>pilosula</i>	Australia
	<i>Manica</i>	<i>rubida</i>	France
	<i>Pogonomyrmex</i>	<i>maricopa</i>	North America
	<i>Strumigenys</i>	sp.	French Guiana
	<i>Daceton</i>	<i>armigerum</i>	French Guiana
	<i>Solenopsis</i>	<i>saevissima</i>	French Guiana
	<i>Myrmica</i>	sp.	France
	<i>Myrmica</i>	<i>lobulicornis</i>	France
	<i>Myrmica</i>	<i>ruginodis</i>	France
	<i>Myrmica</i>	<i>sabuleti</i>	France
	<i>Myrmica</i>	<i>sulcinodis</i>	France
Pseudomyrmecinae	<i>Tetramorium</i>	sp.	France
	<i>Chalepoxenus</i>	<i>muellerianus</i>	France
	<i>Strongylognathus</i>	<i>testaceus</i>	France
	<i>Pseudomyrmex</i>	<i>termitarius</i>	French Guiana
	<i>Pseudomyrmex</i>	<i>tenuis</i>	French Guiana
	<i>Pseudomyrmex</i>	<i>gracillis</i>	French Guiana
	<i>Tetraoponera</i>	sp.	Unknown

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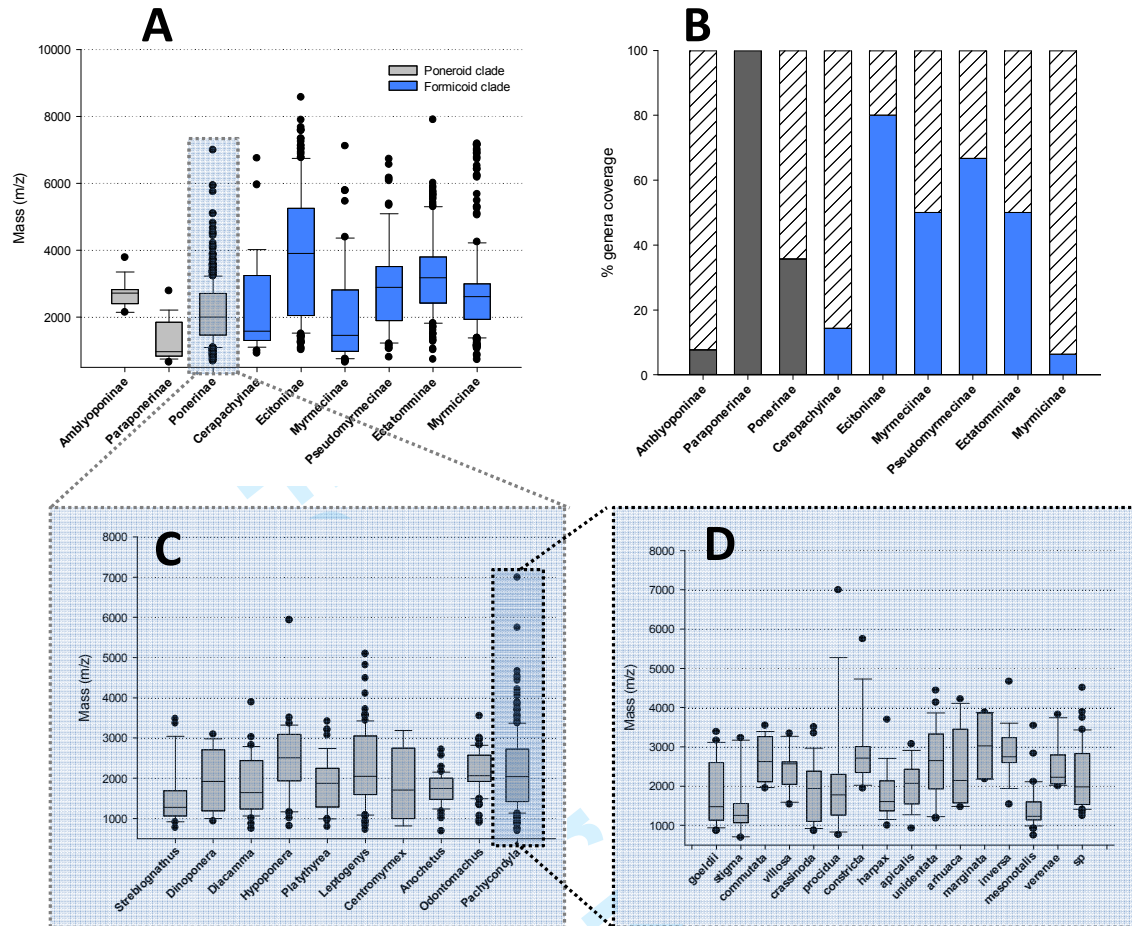


Fig. 1 – MALDI-TOF MS analysis of 82 crude venoms belonging to 9 stinging ant subfamilies from poneroid and formicoid clades. (A) Box-and-whisker plot of the peptide mass distribution of all 82 ant venoms organised by subfamilies. The bottom and top end of each box represent the first and third quartiles, respectively, while the line inside each box represents the median mass. The ends of the whiskers represent the 5–95 percentile range while the black circles represent masses outside the 5–95 percentile range. (B) Diversity coverage (%) of the nine subfamilies of stinging ants studied from poneroid (filled grey bars) and formicoid clades (filled blue bars). The white striped bars represent the percentage of genera not studied within each subfamily. (C) Box-and-whisker plot of the peptide mass distribution of ant venom peptides in the subfamily Ponerinae. (D) Box-and-whisker plot of the peptide mass distribution of ant venom peptides from 16 species of the genus *Pachycondyla*.

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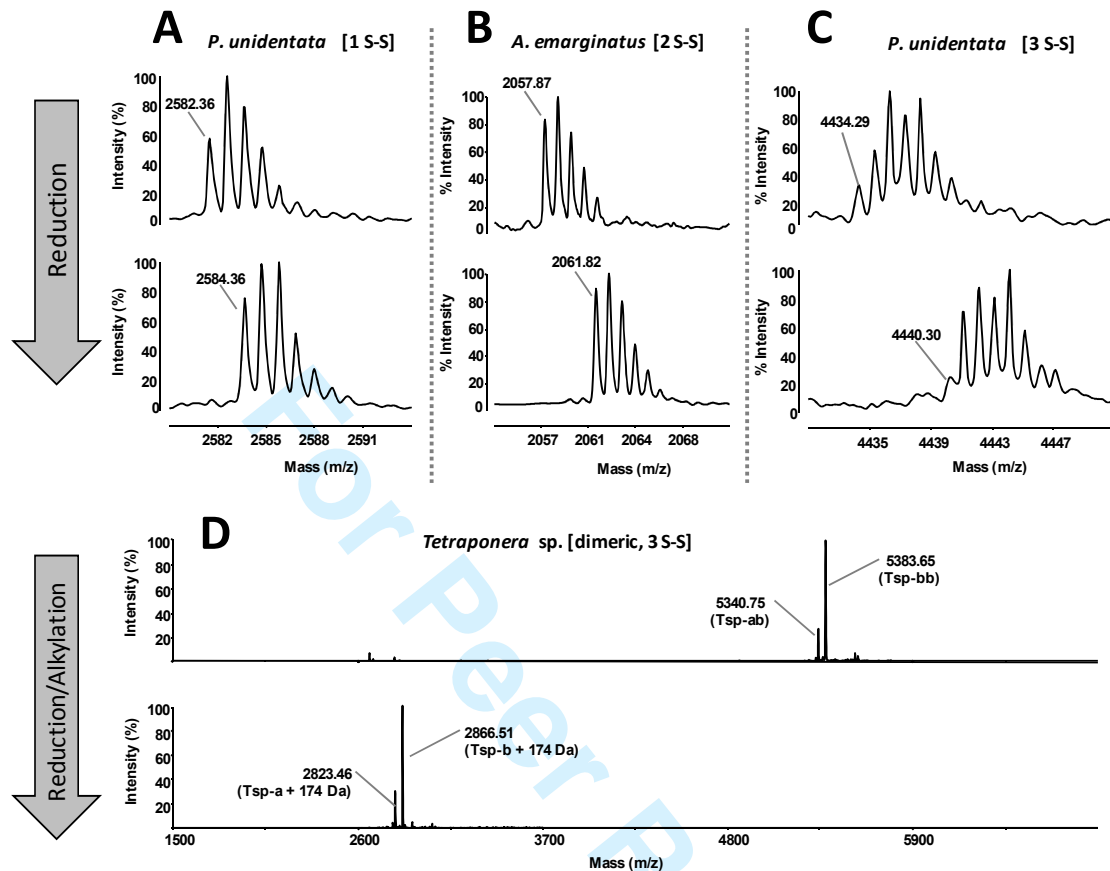


Fig. 2 – Identification of disulfide-bonded peptides in ant venoms. Typical MALDI-TOF MS spectra recorded in reflector mode showing increases in mass of 2 Da (A; *Pachycondyla unidentata*), 4 Da (B; *Anochetus emarginatus*) and 6 Da (C; *Pachycondyla. unidentata*) consistent with the presence of one, two and three disulfide bonds, respectively. Upper panels show native peptide isotopic clusters, while lower panels show the same peptide in reduced form obtained after reduction with DTT. (D) MALDI-TOF mass spectra from *f16* of *Tetraponera* sp. venom before, and after, reduction and alkylation with IAA (R/A). Comparison of spectra before, and after, R/A revealed the presence of the heterodimeric peptide Tsp-ab (5340.75 *m/z*) and the homodimeric peptide Tsp-bb (5383.65 *m/z*). After R/A, peptides Tsp-ab and Tsp-bb disappeared and the mass of the alkylated monomeric Tsp-a (2823.46 *m/z*) and Tsp-b (2866.51 *m/z*) appeared. This is consistent with *S*-carboxymethylation of three cysteines in each monomer.

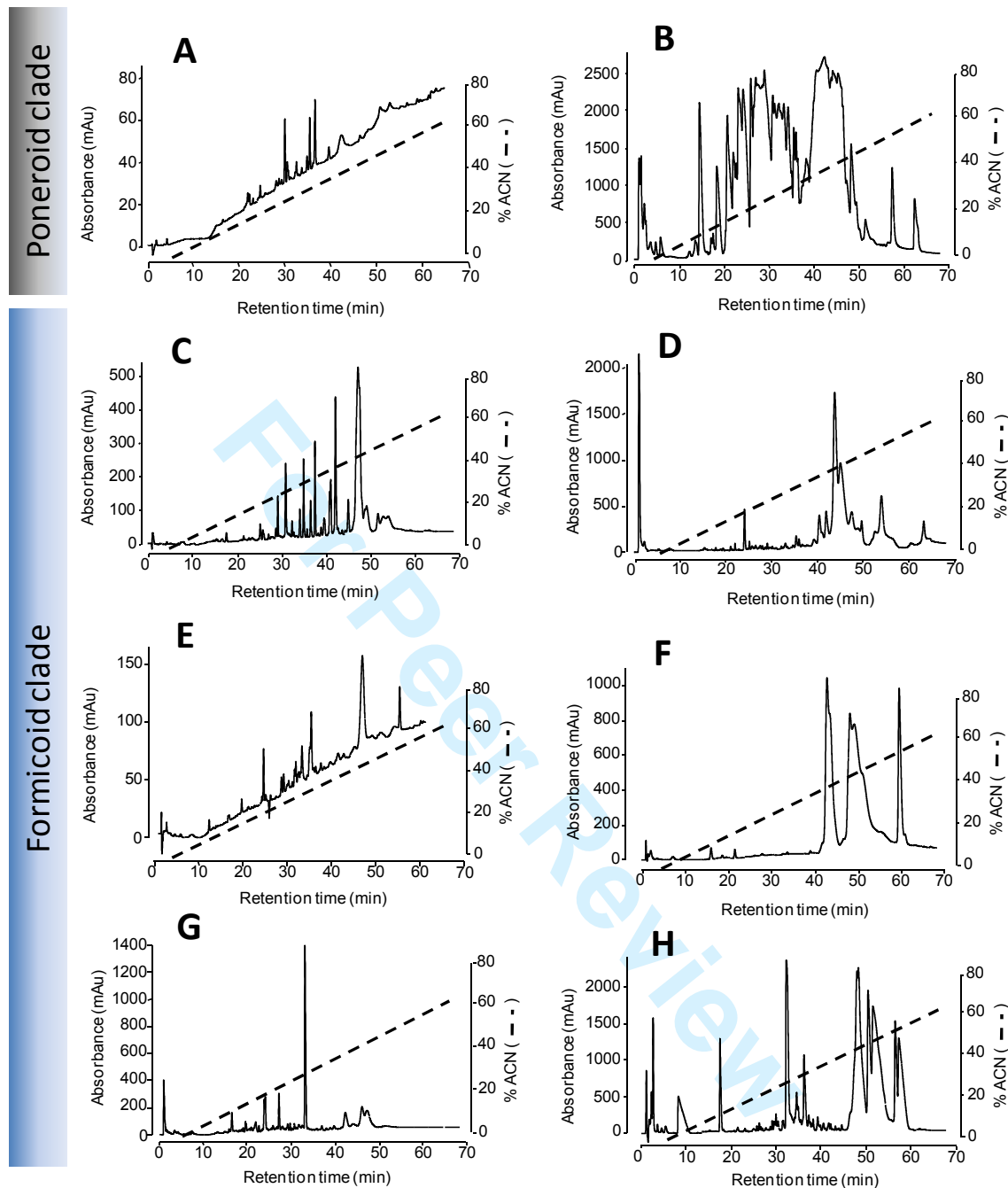
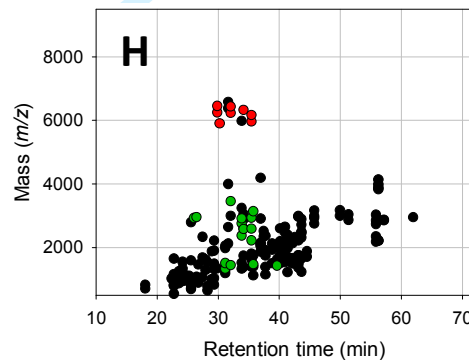
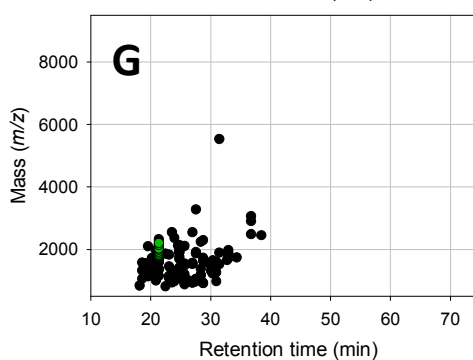
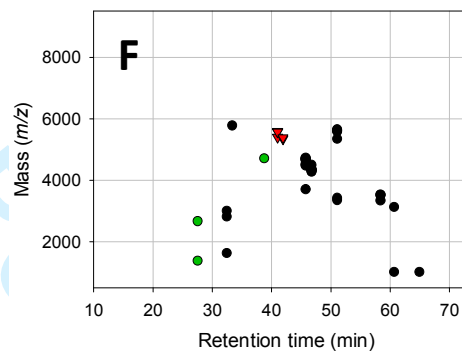
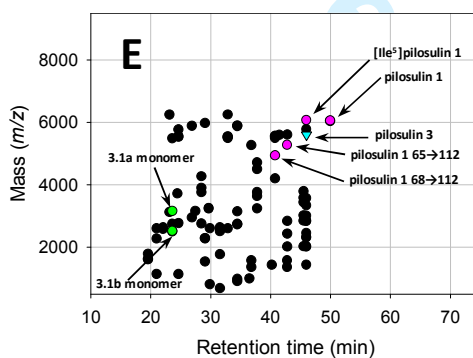
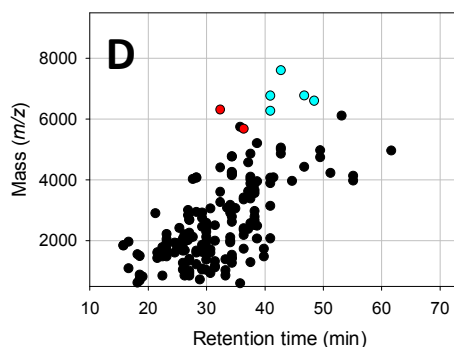
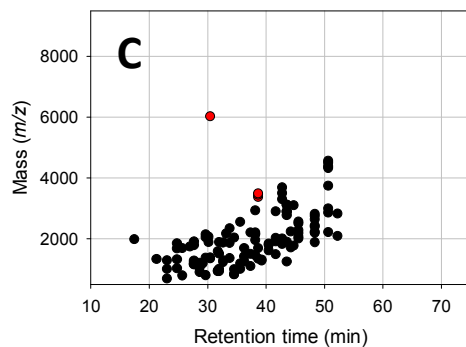
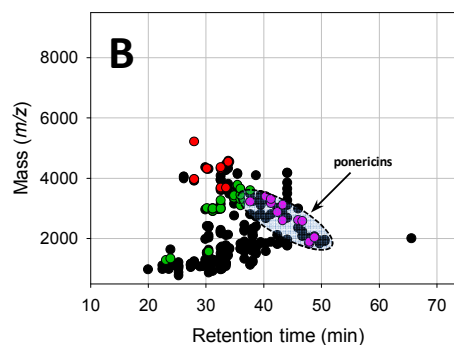
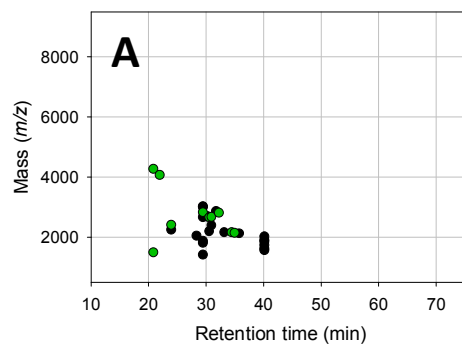


Fig. 3– Representative RP-HPLC chromatograms of formicoid and poneroid ant venoms. Venoms were separated by C18 RP-HPLC using an ACN gradient of 1%/min (black dotted line). Panels show the chromatographic profile of venoms from (A) *Prionopelta* cf. *amabilis* (Amblyoponinae), (B) *Pachycondyla goeldii* (Ponerinae), (C) *Acanthostichus* sp. 1 (Cerapachyinae), (D) *Eciton burchelli* (Ecitoninae), (E) *Myrmecia pilosula* (Myrmeciinae), (F) *Tetraponera* sp. (Pseudomyrmecinae), (G) *Gnamptogenys sulcata* (Ectatomminae) and (H) *Manica rubida* (Myrmicinae).

Poneroid clade



Formicoid clade

Fig. 4 – 2D Landscapes of ant venoms from formicoid and poneroid ants. (A-H) Representative species from eight different subfamilies showing the LC-MALDI-TOF MS venom profiles of (A) *Prionopelta* cf. *amabilis* (Amblyoponinae), (B) *Pachycondyla* *goeldii*

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3 (Ponerinae), (C) *Acanthostichus* sp. 1 (Cerapachyinae), (D) *Eciton burchelli* (Ecitoninae), (E)
4 *Myrmecia pilosula* (Myrmeciinae), (F) *Tetraponera* sp. (Pseudomyrmecinae), (G)
5 *Gnamptogenys sulcata* (Ectatomminae) and (H) *Manica rubida* (Myrmicinae). Black circles
6 indicate peptides without disulfide bonds, green circles represent peptides with one
7 disulfide bond, cyan circles peptides with two disulfide bonds and red circles peptides with
8 three disulfide bonds. Red inverted triangles in the 2D landscape of *Tetraponera* sp. (panel F)
9 show the presence of dimeric peptides with three disulfide bonds while the cyan inverted
10 triangle in the 2D landscape of *M. pilosula* (panel E) represents heterodimeric pilosulin 3
11 reticulated by two disulfide bonds. Purple circles highlight previously characterised pilosulins
12 (panel E) and ponericens (panel B) found in *M. pilosula* and *P. goeldii* venoms, respectively.
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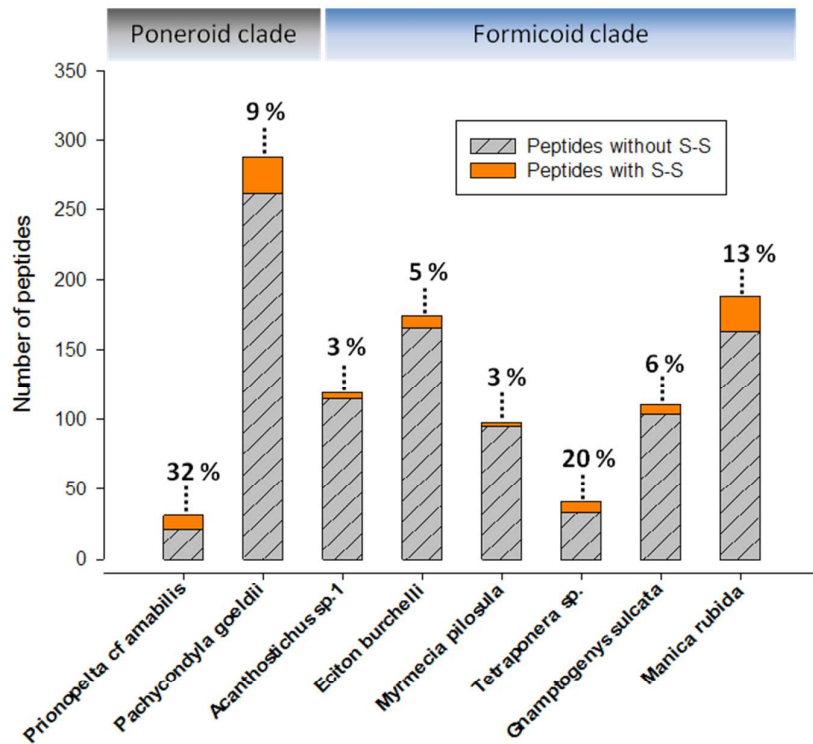


Fig. 5 – Total numbers of peptides in eight representative ant venoms revealed using LC-MALDI-TOF MS. Orange bars show the proportion of disulfide-bonded peptides detected by a mass shift after reduction of each RP-HPLC fraction with DTT. The percentages of disulfide-bonded peptides in whole venoms are indicated above each bar.