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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.

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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

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

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

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

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

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

2. Materials and methods

2.1. Ant Collection and Taxonomy

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

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

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

2.2. Mass spectrometry analysis of crude venoms

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

venoms were acquired in linear or reflector mode and calibrated automatically using the sequence module of the Voyager® Control Software (Applied Biosystems, CA, USA). Five spectra of 50 laser shots per spectrum were accumulated for each sample, based on the acceptance parameters and adequate signal intensity in the 500–10,000 *m/z* range. Mass spectra were collected in positive ion mode with 20 kHz acceleration. Signals below 500 *m/z* were not recorded as they were comprised of mostly matrix-related ion clusters [8].

2.3. RP-HPLC separation

In order to obtain a representative picture of ant venom diversity, we selected one venom each from eight different ant subfamilies to be further analysed by LC-MALDI MS. C18 reversed-phase high-performance liquid chromatography (RP-HPLC) separation of venoms of the following species was therefore conducted: *Prionopelta* cf. *amabilis* (Amblyoponinae), *Pachycondyla goeldii* (Ponerinae), *Acanthostichus* sp. 1 (Cerapachyinae), *Eciton burchelli* (Ecitoninae), *Myrmecia pilosula* (Myrmeciinae), *Tetraponera* sp. (Pseudomyrmecinae), *Gnamptogenys sulcata* (Ectatomminae) and *Manica rubida* (Myrmicinae). These selected ant venoms were fractionated analysed by using an Xterra-C18 5 µm, 2.1 x 100 mm column (Waters, USA) using a gradient of solvent A (0.1% v/v TFA) and solvent B (ACN/0.1% v/v TFA). The percentage of solvent B was modified as follows: 0% for 5 min, 0–60% over 60 min, 60–90% over 10 min and 90–0% over 15 min, at a flow rate of 0.3 mL/min. The eluate was monitored by UV absorbance at 215 nm on a diode-array detector. All analyses were performed on an HP 1100 HPLC system (Agilent, Santa Clara, CA, USA). Peptide elution was monitored in real time and fractions were collected manually for each eluting peak. Each fraction was then dried and reconstituted in 50 µL of 0.1% v/v TFA for offline MALDI-TOF MS analysis and disulfide bond reduction.

2.4. Disulfide bond reduction and alkylation

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

results in a mass increase of 2 Da for each disulfide bond. Thus, by comparing the mass spectra of native and reduced samples the number of disulfide bonds in ant venom peptides can be determined. However, due to the use of a single-stage MALDI-TOF MS instrument, the resolution of the instrument did not permit detection of +2 Da mass differences in peptides with masses above 5kDa. Therefore, venom fractions which contained peptide masses over 5 kDa were also alkylated, following DTT reduction, by incubating the mixture with 50 mM iodoacetic acid (IAA) for 15 min at room temperature in dark. The formation of the S-carboxymethyl derivative of cysteine results in a mass increase of 116 Da for each disulfide bond (58 Da for each cysteine residue) that allows the determination of the number of disulfide bonds in larger venom peptides.

2.5. Mass spectra analysis

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

3. Results

3.2. Crude venom analysis

Crude venoms of the 82 ant species were initially analyzed by MALDI-TOF mass spectrometry and a total of 1396 distinct masses were detected in all venoms after elimination of masses consistent with doubly charged ions, or potassium and sodium adducts. The mean number of peptides detected in crude venom profiles was 17 ± 9 and varied widely, ranging from 4 to

42 in *Chalepoxenus muellerianus* (Myrmicinae) and *Solenopsis saevissima* (Myrmicinae) venoms, respectively.

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

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

average amino acid, averagine, calculated with the formula $C_{4.9384}H_{7.7583}N_{1.3577}O_{1.4773}S_{0.0417}$ [50]. This finding is similar to conotoxins that are typically between 10 and 30 amino acids in length [51] but is in contrast to spider, scorpion and snake venom peptide toxins that are normally between 40 and 100 amino acids [52].

3.3. Disulfide bond mapping in crude venoms

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

Only 32 peptides with 1 disulfide bond were detected in 14 crude venoms (*Pachycondyla commutata*, *P. mesonotalis*, *P. unidentata*, *Odontomachus hastatus*, *O. scalptus*, *Anochetus horridus*, *A. cf. diegensis*, *Streblognathus aethiopicus*, *Prionopelta cf. amabilis*, *Ectatomma brunneum*, *Myrmecia rufinodis*, *M. similima*, *Pogonomyrmex maricopa*, *Manica rubida*); 17 peptides with 2 disulfide bonds were detected only in venoms from the genus *Anochetus* (*A. horridus*, *A. cf. diegensis* and *A. emarginatus*); and 1 peptide with 3 disulfide bonds was detected in *P. unidentata* venom. From our sample survey, more disulfide-linked peptides were detected in venoms from the poneroid clade (42 disulfide-bonded peptides) than in the formicoid clade (8 disulfide-bonded peptides). Overall, 33 disulfide-bonded peptides were found in Ponerinae venoms plus 9 in Amblyoponinae venoms (all poneroids), whereas only 1 disulfide-bonded peptide was found in Ectatomminae, 2 in Myrmeciinae and 5 in Myrmicinae venoms (from the formicoid clade). This may indicate greater structural diversity in the Ponerinae, although a larger venom sample set encompassing more species would be needed for any definitive conclusion to be drawn.

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

in venom of *Prionopelta* cf. *amabilis* (Amblyoponinae) were structured by 1 disulfide bond. This suggests that our study, although sampling a broad range of ant subfamilies, cannot reveal the full extent of ant peptidome diversity. The vast number of stinging ant species (ca. 9,000 species) may reveal other peptide classes, structures and pharmacological properties. This is because specific genera like *Anochetus* and *Prionopelta* may have evolved different venoms based on atypical structural motifs.

3.4. LC-MALDI analysis and 2D landscapes

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

The resultant chromatographic profiles showed few similarities between the various subfamilies (Fig. 3) and varied in their complexity. This is reflected by the number of fractions collected from the chromatograms. A total of 27 fractions were collected from the least complex venom (*Tetraponera* sp.) and 61 from the most complex venom (*Eciton burchelli*). The overall complexity and range of elution patterns observed in ant venoms is parallel to that observed with RP-HPLC profiles of other venomous arthropods such as

Australian funnel-web spiders [10]. The eight ant venoms that were fractionated showed peptides eluting in a broad range of hydrophobicity. The most hydrophilic fraction eluted at 10.8% ACN (Fraction *f*1 from the venom of *Eciton burchelli* at 15.8 min) and the most hydrophobic fraction eluted at 60% ACN (*f*43 from the venom of *Pachycondyla goeldii* at 65 min). Venom of *Prionopelta cf. amabilis* eluted over a narrow hydrophobicity range (15.9–35.2% ACN) while both *Manica rubida* and *Pachycondyla goeldii* venoms eluted in a broad hydrophobicity range (13.1–57% ACN and 15–60% ACN, respectively). This highlights the broad physicochemical diversity among ant venom peptides, suggesting that peptides with different structural scaffolds or widely differing amino acid compositions are present.

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

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

371
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*, *Tetraponera* 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 (*Tetraponera* 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 *Tetraponera* 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

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

4. Discussion

Ant venoms evolved bioactive peptides in order to disrupt multiple biological targets and permit the capture of arthropod prey, deter predators, communicate with other members of the colony and also act as antimicrobials. It is therefore not surprising that the present study of the ant venom peptidome has revealed enormous variation in the complexity of ant venoms highlighting the potentially diverse pharmacologies and functions of ant venom peptides. It is now well established from other taxonomic groups [7-8] that venoms are highly complex, containing up to 1000 peptides, and that many peptide classes as well as numerous isoforms in each class combine into a complex biochemical cocktail. Our investigation of ant venoms reveals that a wide range of ants produce venoms with a peptidic composition as complex as those of spiders, scorpions or cone snails as previously demonstrated in the venom of the ponerine ant *Dinoponera quadriceps* [28]. LC-MALDI-TOF mass fingerprinting of a wide range of poneroid and formicoid ant venoms detected total numbers of peptides ranging from 31 to 288 peptides. Indeed, venoms of *Pachycondyla* (Ponerinae) ants seem to be particularly rich in peptide components and the total number of peptides appear to be as complex as the previously described venom from the related ponerine ant *Dinoponera quadriceps* [28]. Based on the present study, we would estimate that ant venoms contain on average ca. 130 unique peptides. If we assume a total of ca. 9,000 stinging ant species, we can calculate the total number to be more than 1 million peptides in all stinging ant venoms. However, this number is probably an under-estimate, as this does not take into account intra-species variations observed in ant venoms [28]. Moreover, cryptic ant species also contribute to a hidden peptide diversity as the venom

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peptide profile can vary among cryptic ant species [21]. Thus, many ant species are still undiscovered particularly within tropical areas and the total number of ants could reach 25,000 species (currently ca. 13,000 described species) [58].

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

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

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

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

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

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

small sizes of ant venom peptides make sequencing and synthesis of these toxins relatively facile therefore facilitating characterisation of their pharmacological targets. Ant venoms may therefore provide a novel source of bioactive peptides to develop drug and 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	
	Heteroponerinae	3	0		24	0	
leptanilloid	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>	Ponerinae	poneroid		
	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>				
	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>				
	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>	Amblyoponinae			
	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>				
	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>	Myrmicinae		formicoid	
	2052.93	2054.95	<i>Pogonomyrmex maricopa</i>				
	2489.00	2491.12	<i>Pogonomyrmex maricopa</i>				
	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>	Ponerinae	poneroid		
	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>				
	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>	cf. <i>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>prociua</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>	cf. <i>ruginodis</i>	French Guiana
	<i>Anochetus</i>	<i>emarginatus</i>	French Guiana
	<i>Anochetus</i>	cf. <i>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>Hypoconera</i>	sp. 1	French Guiana
	<i>Hypoconera</i>	sp. 2	French Guiana
	<i>Hypoconera</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
	<i>Nomamyrmex</i>	<i>hartigi</i>	French Guiana
Ectatomminae	<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
Myrmeciinae	<i>Gnamptogenys</i>	sp. 1	French Guiana
	<i>Gnamptogenys</i>	sp. 2	French Guiana
	<i>Myrmecia</i>	<i>gulosa</i>	Australia
	<i>Myrmecia</i>	<i>rufinodis</i>	Australia
Myrmicinae	<i>Myrmecia</i>	<i>simillima</i>	Australia
	<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
	<i>Tetramorium</i>	sp.	France
	<i>Chalepoxenus</i>	<i>muellerianus</i>	France
Pseudomyrmecinae	<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>Tetraponera</i>	sp.	Unknown

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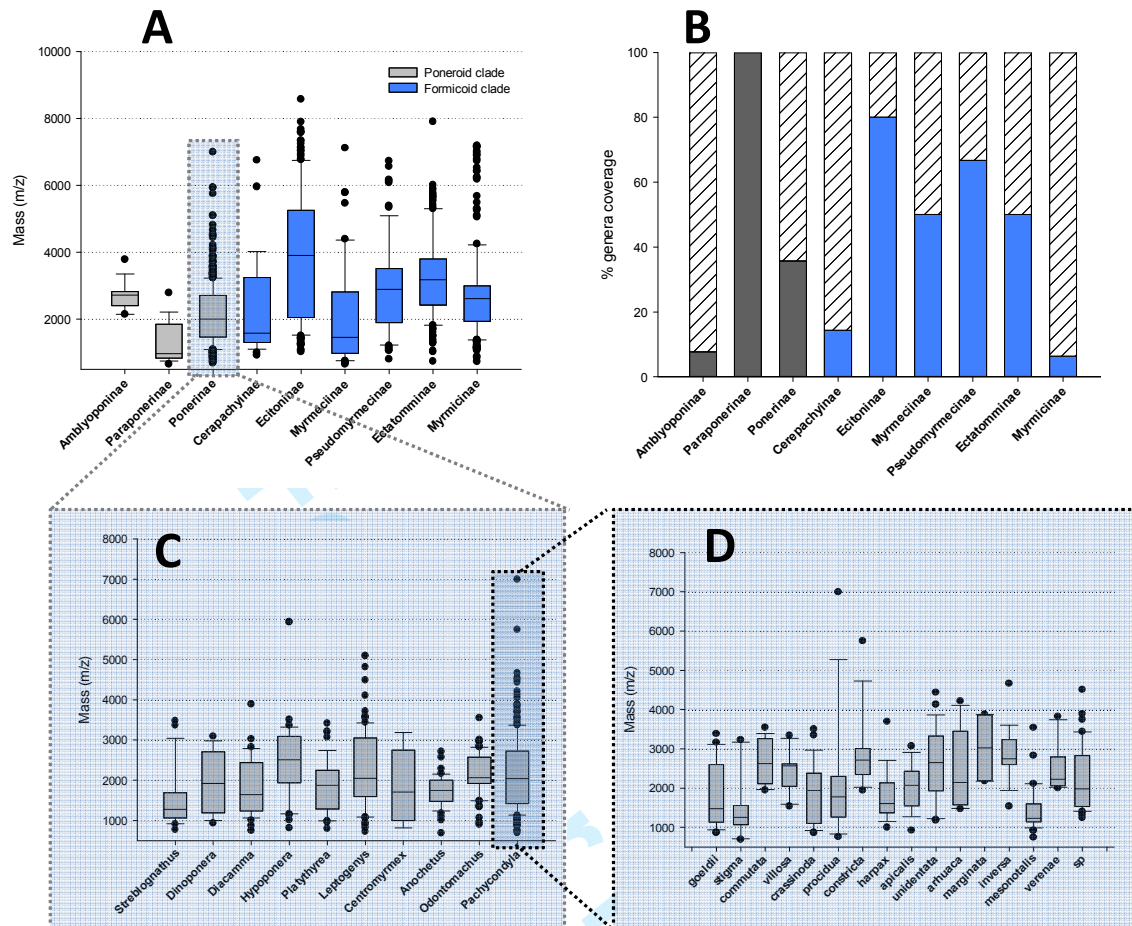


Fig. 1 – MALDI-TOF MS analysis of 82 crude venoms belonging to 9 stinging ant subfamilies from poneroid and formicoid clades. (A) Box-and-whisker plot of the peptide mass distribution of all 82 ant venoms organised by subfamilies. The bottom and top end of each box represent the first and third quartiles, 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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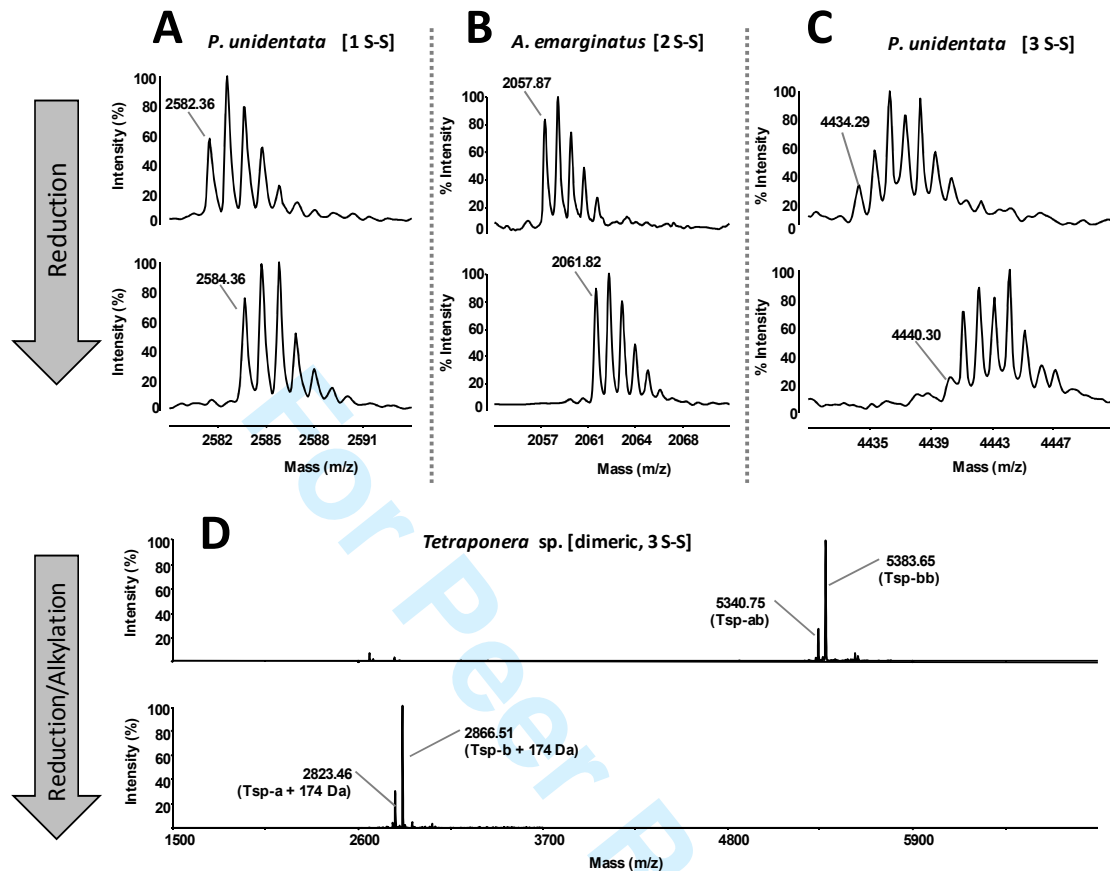


Fig. 2 – Identification of disulfide-bonded peptides in ant venoms. Typical MALDI-TOF MS spectra recorded in reflector mode showing increases in mass of 2 Da (A; *Pachycondyla unidentata*), 4 Da (B; *Anochetus emarginatus*) and 6 Da (C; *Pachycondyla. unidentata*) consistent with the presence of one, 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.

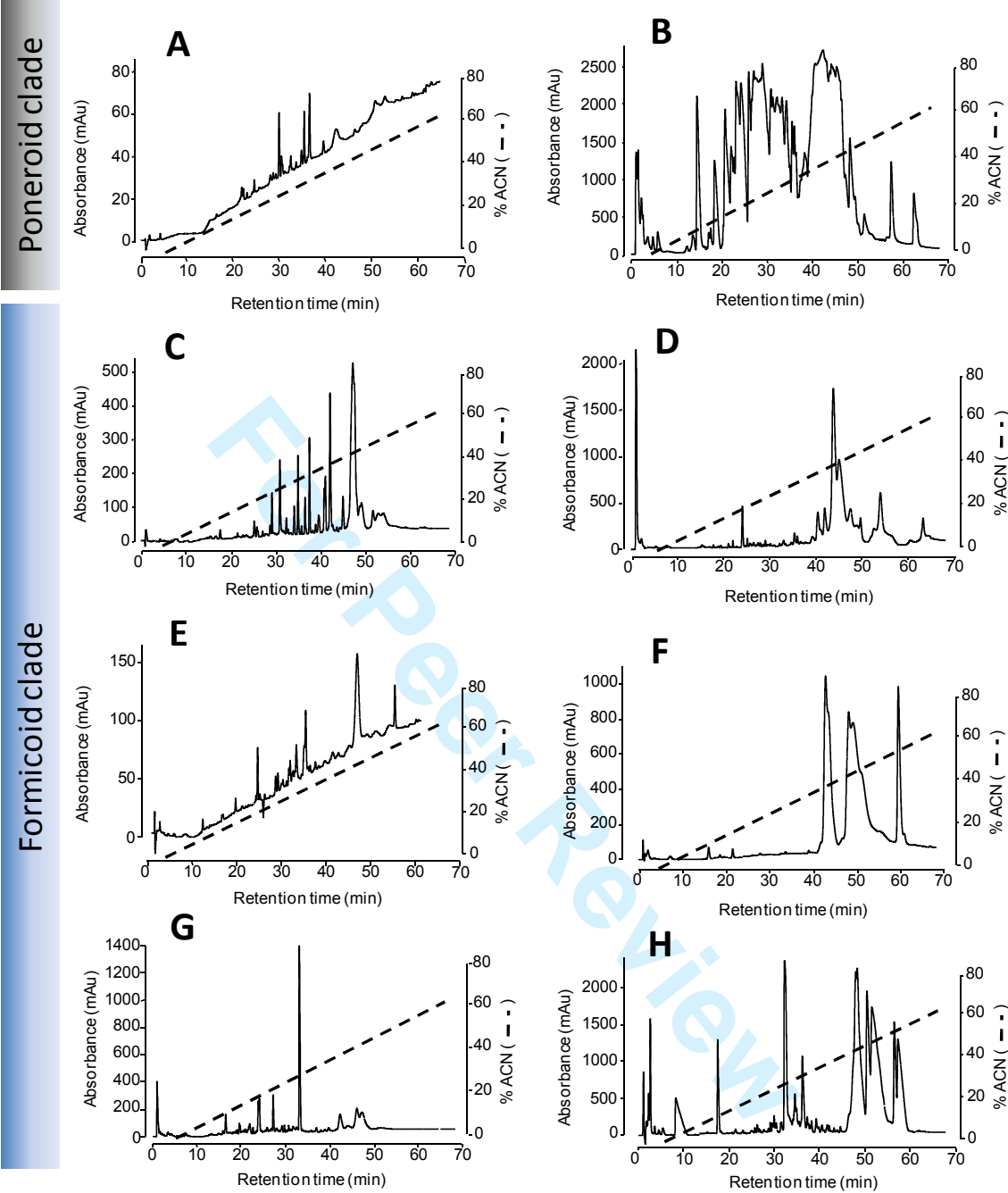


Fig. 3– Representative RP-HPLC chromatograms of formicoid and poneroid ant venoms. Venoms were separated by C18 RP-HPLC using an ACN gradient of 1%/min (black dotted line). Panels show the chromatographic profile of venoms from (A) *Prionopelta* cf. *amabilis* (Amblyoponinae), (B) *Pachycondyla goeldii* (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

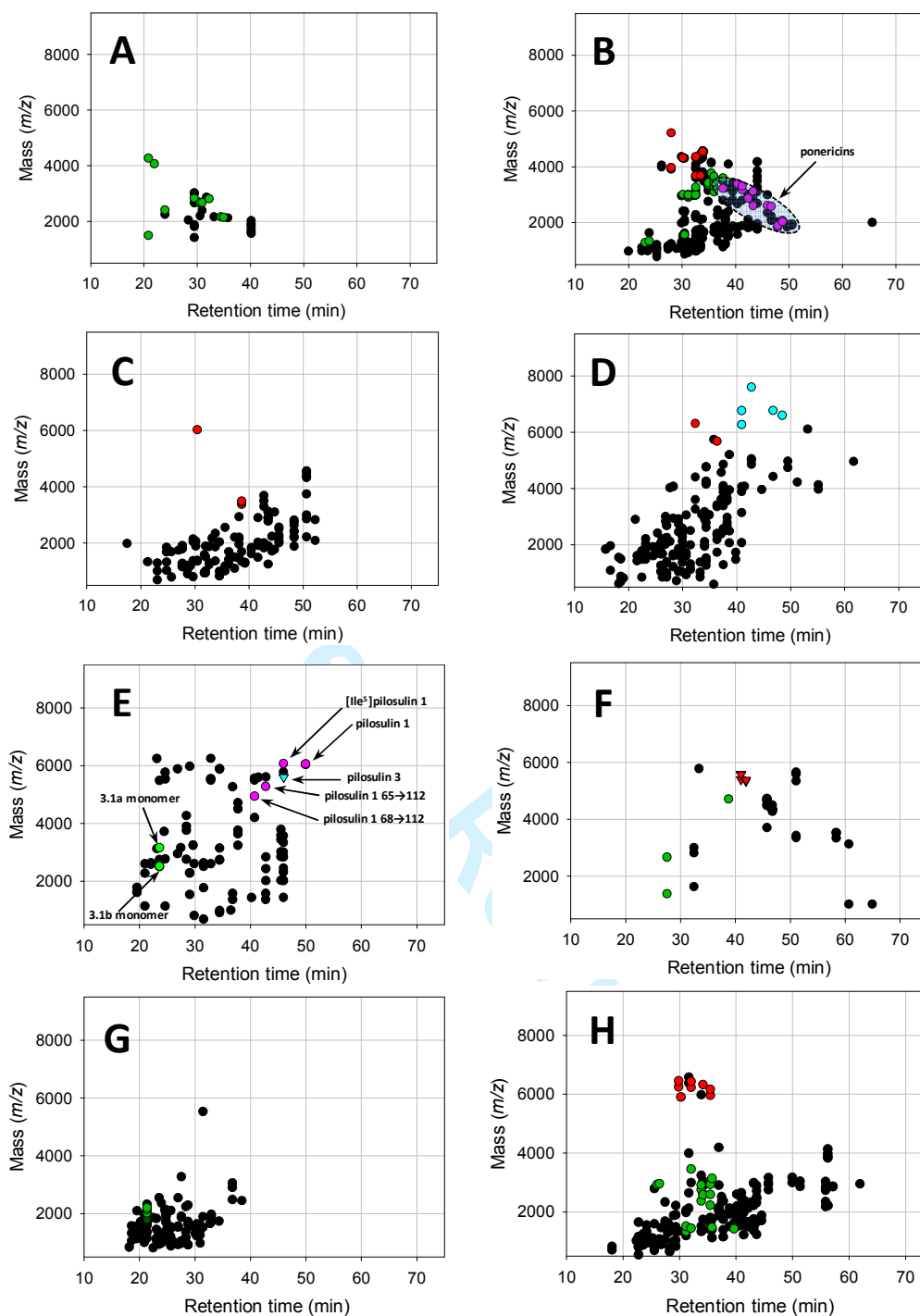


Fig. 4 – 2D Landscapes of ant venoms from formicoid and poneroid ants. (A-H) Representative species from eight different subfamilies showing the LC-MALDI-TOF MS venom profiles of (A) *Prionopelta* cf. *amabilis* (Amblyoponinae), (B) *Pachycondyla goeldii*

(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). Black circles indicate peptides without disulfide bonds, green circles represent peptides with one disulfide bond, cyan circles peptides with two disulfide bonds and red circles peptides with three disulfide bonds. Red inversed triangles in the 2D landscape of *Tetraponera* sp. (panel F) show the presence of dimeric peptides with three disulfide bonds while the cyan inversed triangle in the 2D landscape of *M. pilosula* (panel E) represents heterodimeric pilosulin 3 reticulated by two disulfide bonds. Purple circles highlight previously characterised pilosulins (panel E) and ponerinicins (panel B) found in *M. pilosula* and *P. goeldii* venoms, respectively.

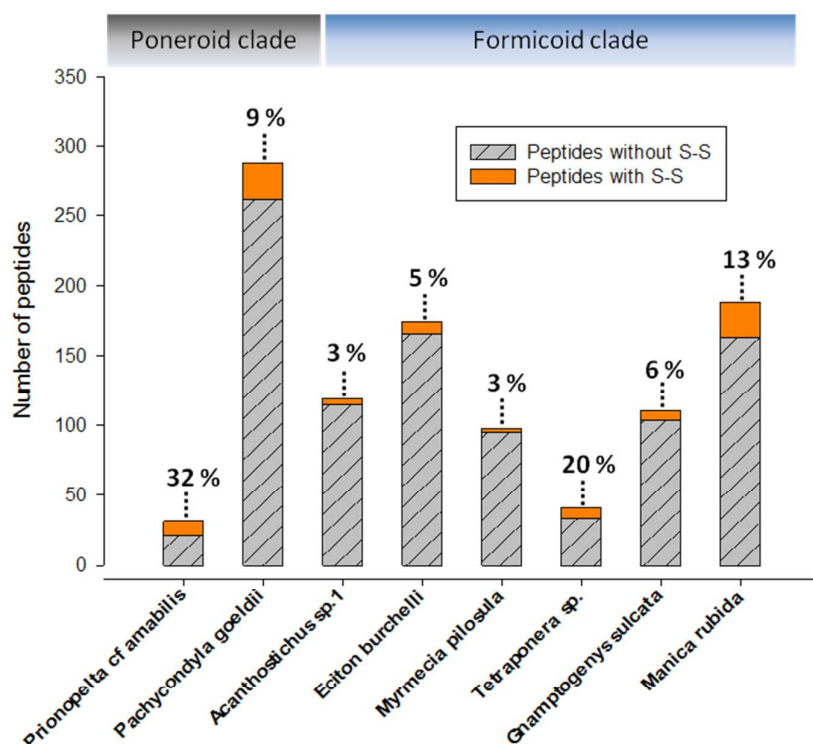


Fig. 5 – Total numbers of peptides in eight representative ant venoms revealed using LC-MALDI-TOF MS. Orange bars show the proportion of disulfide-bonded peptides detected by a mass shift after reduction of each RP-HPLC fraction with DTT. The percentages of disulfide-bonded peptides in whole venoms are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