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Axel Touchard[1,2,†] Samuel D. Robinson[3], Hadrien Lalagüe[1], Steven Ascoët[4], Arnaud Billet[4], Alain Dejean[1,5], Nathan J. Téné[4], Frédéric Petitclerc[1], Valérie Troispoux[6], Michel Treilhou[4], Elsa Bonnafé[4], Irina Vetter[3,7], Joel Vizueta[8], Corrie S. Moreau[2], Jérôme Orivel[1,*], Niklas Tysklind[6,*]

[1]CNRS, UMR Ecologie des forêts de Guyane – EcoFoG (AgroParisTech, CIRAD, INRAE, Université de Guyane, Université des Antilles), Campus Agronomique, BP 316, 97379 Kourou Cedex, France.

[2]Department of Entomology, Cornell University, Ithaca, New York, USA.

[3]Institute for Molecular Bioscience, The University of Queensland, QLD 4072, Australia.

[4]Equipe BTSB-EA 7417, Université de Toulouse, Institut national universitaire Jean-François Champollion, Place de Verdun, 81012, Albi, France.

[5]Centre de Recherche sur la Biodiversité et l'Environnement, Université de Toulouse, CNRS, Toulouse INP, Université Toulouse 3 – Paul Sabatier (UPS), Toulouse, France.

[6]INRAE, UMR Ecologie des forêts de Guyane - EcoFoG (AgroParisTech, CIRAD, CNRS, Université de Guyane, Université des Antilles), Campus Agronomique, BP 316, 97379 Kourou Cedex, France.

[7]School of Pharmacy, The University of Queensland, Woolloongabba, QLD 4102, Australia.

[8]Villum Centre for Biodiversity Genomics, Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Copenhagen, Denmark.

[*]These authors contributed equally to this work.

†Axel Touchard (A.T.)
Email: axel.touchard2@gmail.com (A.T.)

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Abstract

Stinging ants have diversified into various ecological niches, and selective pressures may have contributed to shape the composition of their venom. To explore the drivers underlying venom variation in ants, we sampled 15 South American rainforest species and recorded a range of traits, including ecology, morphology, and venom bioactivities. Principal component analysis of both morphological and venom bioactivity traits reveal that stinging ants display two functional strategies where species have evolved toward either an exclusively offensive venom or a multifunctional venom. Additionally, phylogenetic comparative analysis indicates that venom function (predatory, defensive, or both) and mandible morphology correlate with venom bioactivity and volume. Further analysis of the venom biochemistry of the 15 species revealed switches between cytotoxic and neurotoxic venom compositions among species. Our study supports an evolutionary trade-off between the ability of venom to deter vertebrate predators and to paralyze insect prey which are correlated with different venom compositions and life history strategies among Formicidae.

1. Introduction

Most ants have venom, the composition of which can vary considerably among lineages; some species have formic acid or alkaloid-based venoms, while the venoms of most stinging species are peptidic [1]. Among different lineages of stinging ants, venoms can exhibit very different peptide toxin profiles [2,3], presumably in response to distinct functional uses. The Formicidae have radiated into diverse ecological niches [4], and numerous evolutionary forces may have contributed to the shaping of their venoms. Here, we evaluate several ecological factors that can be considered as potential drivers of venom evolution in ants:

(*i*) Foraging activities of predatory ants range from subterranean to canopy habitats. We hypothesized that predatory venom efficacy is more important for arboreal ants that need to subdue prey rapidly to prevent it from falling to the ground, leading to highly paralytic venom. Previous research on ponerine ants suggested that arboreal habits may influence the efficacy of venom in capturing prey [5].

(*ii*) Diet is often a potent driver of venom evolution in predatory organisms [6]. Many predatory ants use their venom to capture a diversity of prey; however, several species or lineages are stenophagous (i.e. prey exclusively on a restricted group of arthropods) [7]. Venoms of stenophagous ants may therefore be more effective on their intended prey, while greater taxonomic diversity of prey would lead to more complex venom compositions [8–10].

(*iii*) As most stinging ants also use their mandibles to subdue their prey before delivering the paralyzing sting, mandible shape varies widely, which could be linked to venom composition. The

morphology of the mandibles of some predatory ants is indeed specialized to the shape of the prey [7,11], while trap-jaw ants use spring-loaded mandibles that snap shut on prey with high speed and force [12]. The presence of specialized trap-jaw mandibles, which enable efficient physical capture of prey, might be negatively correlated with venom amount and efficacy to paralyze insect prey as an ecological trade-off.

(*iv*) Ants that live in mutualistic association with myrmecophytes (e.g. acacia ants) use their venom not for predation, but for fierce protection of the host plant [13], while in contrast, some stinging species lack aggression toward potential predators [14]. These non-aggressive ants exhibit thanatosis (i.e. feigning death) [15], escape behavior [16], or rely on morphological attributes such as spines as a deterrent rather than using their sting against vertebrate predators [17]. Venom function, whether predatory, defensive, or both, is therefore a putative driver of venom composition in ants. We hypothesize that selective pressures for defense have led ants to produce large amounts of venom comprising painful toxins, while ants that primarily use their venom to subdue prey would deliver a small amount of highly effective venom to paralyze insects.

We designed a phylogenetically nested sampling of South American rainforest ant genera, each with several congeneric species with contrasting ecological traits. The genera were selected at different phylogenetic distances: sister genera, genera within the same subfamily, and genera from different families, to evaluate if the obtained signals were conserved across the phylogeny. Our sampling was mainly based on foraging activity: arboreal vs terrestrial. The effect of foraging activity on venom was tested in three genera of the subfamily of Ponerinae (i.e. *Anochetus*, *Odontomachus,* and *Neoponera*) and in a fourth non-ponerine genus, *Pseudomyrmex.* Ponerinae are a basal lineage of ants while Pseudomyrmecinae represent a more derived subfamily. We also extended our sampling to two other arboreal ant species (*Daceton armigerum* and *Paraponera clavata*), since previous studies had highlighted unusual venom compositions [2,18]. This sampling also gave us the opportunity to gain insight into how other potential ecological factors may contribute to the shaping of ant venom. Two species were presumed to be stenophagous: *Neoponera commutata* is a known specialized termite predator [19] and *Anochetus emarginatus* is also suspected to be a termite specialist [20,21] in contrast with their respective congeners included here. The inclusion of *D. armigerum* allowed testing the convergent effects of trap-jaw mandibles on venom evolution with the sister genera *Odontomachus* and *Anochetus* [12]. *Paraponera clavata*, known as the bullet ant and notable for its painful defensive sting [22], was also included in this panel as a venom that has evolved to effectively repel vertebrate predators. Within the genus *Pseudomyrmex,* we used ground-dwelling (*P. termitarius*) and arboreal (*P. gracilis*) predatory species and compared them with obligate plant-ant species (*P. viduus* and *P. penetrator*), which never use their venoms for predation [23], to examine the effects of relaxed selection pressures for predatory capacity on venom diversity.

To date, no studies have integrated ecological traits, biochemical composition, and bioactivities in a phylogenetic framework to explore the factors that lead to distinct venom compositions in ants and, more broadly, very few in other venomous lineages [24]. To evaluate the impact of the abovementioned ecological traits on ant venoms we analyzed stinging behavior, diet, a suite of morphological traits, venom activities, and venom composition while controlling for phylogenetic relationships. Among morphological traits we measured length of the sting and mandibles since ants use both to capture prey and to defend against predators leading to the hypothesis that a long sting would be associated with a defensive function, while long mandibles would allow for better seizure of prey. We evaluated the volume of the whole venom reservoir to provide information on the metabolic cost of venom production. Venom activities on blowflies and mouse sensory neuronal cells informed on venom efficacy to subdue insects and deter

vertebrates. To provide insights into the mechanism of action of the venoms, we evaluated their cytotoxicity against *Drosophila* cells using two assays that measure the effect on cell metabolism and membrane cell integrity. Finally, to understand the biochemical mechanisms underlying the observed variations in venom activities and modes of action, we examined the venom composition of each of the 15 species and discussed the results in light of the life history of each ant species.

2. Material and Methods

(a) Ants and venom samples preparation

Live specimens of worker ants from different colonies for each species were collected in French Guiana. Two additional ant species were originally included in the sampling design (*A. targionii* and *P. tenuis*) but could not be investigated because of the low amount of venom obtained. To identify transcripts involved in venom production, we aimed to generate whole-body (i.e. head and thorax) and venom gland transcriptomes for each species, and then, subtract transcripts expressed in the whole-body transcriptome from those in the venom gland, to identify those transcripts expressed largely in venom glands. For each species we dissected: 1) both venom glands and venom reservoirs from 100 live workers per species in ultrapure water and immediately placed in 1 mL of RNAlater™ (Thermo Fisher Scientific, Waltham, MA, USA); and 2) the head and thorax of 2-3 workers in 1 mL of RNAlater™. Samples were stored at –80°C prior to RNA extraction. Crude venom samples were prepared by dissecting ant venom reservoirs in ultrapure water, then pooled in 10% acetonitrile (ACN) in ultrapure water and stored at –20°C prior to freeze-drying. Venom samples were then loaded onto a 0.45 µm Costar® Spin-X tube filter (Corning Incorporated, Corning, NY, USA) and centrifuged at 12,000 g for 3 min to remove tissues from the venom apparatus. Filtered venom samples were then lyophilized, weighed, and stored at –20°C until further use. For each species we also estimated the venom yield, i.e. the weight of freeze-dried venom from a pool of several individual venom reservoirs divided by the number of dissected workers. This value provides an estimate of the maximum amount of venom that an individual worker would be capable of delivering.

(b) Venom composition and transcripts classification

To characterize the venom composition of each species we employed a transcriptomic approach coupled with mass spectrometry to validate the presence of peptides in the venom (the proteo-transcriptomic method is described in electronic supplementary material, supporting methods). From the transcriptomes generated, the annotations were manually curated focusing on transcripts coding for toxins, with consideration of gene expression levels in venom glands and in the body. We also selected and examined additional transcripts based on precursor similarities to known toxin peptides. A total of 465 transcripts encoding putative toxins were retained for subsequent analyses (electronic supplementary material, Figure S1 and Dataset S1). Proteomic data were generated from the mass spectrometry fragmentation spectra using PEAKS software [25], with the transcriptome of each species implemented as a database for peptide sequence assignment. Positive matches of proteomics data with transcripts allowed us to confirm 305 peptide sequences. Total ion chromatograms were also generated with crude venoms and the LC-MS profile annotated (electronic supplementary material, Figure S2-3). Peptide transcripts were clustered into five gene clades (i.e. cysteine-rich poneritoxin, ponericin-like, pseudomyrmeciitoxin (PSDTX), ICK-PONTX, dimeric myrmicitoxin (MYRTX)) according to the predictive signal sequence (electronic supplementary material, Figure S4). To define gene clades, we used an approach based on similarity of signal parts. Signal parts were predicted

using SignalP - 6.0 server [26] and then aligned using the Muscle program in MEGA-X [27]. A pairwise distance matrix between sequences was extracted from the multiple alignments and used for HCA clustering using the hclust function with the ward method from the R package "stats". Transcripts were further manually classified into families based on the similarity of the amino acid sequences of the whole precursors (signal, pro-, and mature regions). For each family, multiple alignments of full-length precursors were generated using the Muscle program in MEGA-X version 10.1.8 [27] and edited using Jalview version 2.11.2.7 [27,28]. We classified the transcripts into 62 peptide families and 3 enzyme toxins (i.e. phospholipase A_1, phospholipase A_2, and venom allergen 3) (electronic supplementary material, Figure S5-12). The full list of transcripts expressed in venom glands with a TMM greater than 100, the identified toxin precursor sequence, the predicted mature part, the family assignment, and PEAKS results can be found in Dataset S1. The venom composition of *Pa. clavata,* previously published by Aili *et al* [18] has been included in our analysis. A total of 14 toxin families (i.e. families 23, 26, 29, 33, 34, 35, 37, 49, 52, 54, 56, and 59) were not included in the venom composition analysis for clustering because no transcript sequences could be confirmed by mass spectrometry, yet they were retained in the sequence alignments. For venom clustering, a Bray-Curtis distance matrix based on the relative expression of the toxin family was generated using the veggdist function from the R package "vegan" [29], followed by hierarchical clustering analysis (HCA) using the hclust function with the full method from the R package "stats" [30]. The dendlist function from the R package "dendextend" [31] was used to plot and align the species phylogeny tree with the venom composition HCA cladogram. The final Figure 2 was edited in GraphPad Prism v10.0.3.

(c) Morphological traits

Six morphological traits were measured on up to 13 randomly selected workers per species. Measurements were made using an ocular micrometer accurate to 0.01 mm mounted on a Leica M80 or Leica S9E stereomicroscope (Leica Microsystems, Heerbrugg, Switzerland). The traits considered were Weber's length (i.e., the diagonal length of the mesosoma in profile view, which is a proxy for worker size [32], head length, mandible length, sting length, venom reservoir length, and venom reservoir width. We estimated the venom reservoir volume using the standard ellipsoid formula; π/6 (venom reservoir length × venom reservoir width^2). For analysis we used size/volume-corrected ratios calculated as follows: mandible proportion (mandible length / head length), sting proportion (sting length / Weber's length), venom reservoir proportion (venom reservoir volume / Weber's length^3).

(d) Neuronal cell assays

As a proxy for pain in vertebrates, we assessed the potency of each of the 15 venoms to activate mammalian neuronal cells, defined as an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the F11 (mouse neuroblastoma × rat dorsal root ganglion (DRG) neuron hybrid) cell line. F11 were maintained on Ham's F12 media supplemented with 10% FBS, 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine (Hybri-Max, Sigma Aldrich). 384-well imaging plates (Corning, Lowell, MA, USA) were seeded 24 h prior to calcium imaging, resulting in ~90% confluence at the time of imaging. Cells were loaded for 30 min at 37°C with Calcium 4 assay component A in physiological salt solution (PSS; 140 mM NaCl, 11.5 mM D-glucose, 5.9 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 10 mM HEPES) according to the manufacturer's instructions (Molecular Devices, Sunnyvale, CA). Ca^{2+} responses were measured using a FLIPRPenta fluorescent plate reader equipped with a CCD camera (Ex: 470 to

490 nm, Em: 515 to 575 nM) (Molecular Devices, Sunnyvale, CA). Signals were read every second for 10 s before, and 300 s after, the addition of venoms (in PSS supplemented with 0.1% BSA). The pain-causing capacity of a given species' venom is a product of both the venom potency and amount of venom able to be delivered. By dividing the average venom yield (µg) by venom potency (µg/mL) we calculated a value we refer to as the nocifensive capacity for each species.

(e) Insect activity assays

To assess the potency of venoms to paralyze and to kill invertebrate prey, we injected different doses of crude venom into the blowfly *Lucilia caesar* (the average mass of flies was 19 ± 2 mg). Blowfly larvae (*Lucilia caesar*) were purchased from a fisheries shop (Euroloisir81, Lescure-d'Albigeois, France) and kept at 25°C until hatching. Flies 1-4 days after hatching were used for injection assays. Blowfly assays were done through lateral intrathoracic injection of 1 µL of venom dissolved in ultra-pure water at various concentrations using a fixed 25-gauge needle attached to an Arnold hand microapplicator (Burkard Manufacturing Co., Ltd., Rickmansworth, UK) with a 1.0 mL Hamilton Syringe (1000 Series Gastight, Hamilton Company, Reno, NV, USA). Then, the blowfly was placed in an individual 2 mL tube containing 15 µL of 5% glucose solution. Paralysis was monitored at 1 h and 24 h post-injection, while lethality was monitored at 24 h. Flies that did not display any signs of movement dysfunction were considered unaffected, otherwise they were recorded as paralyzed. Flies were deemed dead if they did not respond at all to tweezer mechanical stimulation when observed under a dissecting microscope. Ten flies were used for each toxicity experiment and for the corresponding control (ultrapure water solution). Each dose was repeated three times. Dose–response data were analyzed to determine a half maximal paralytic dose (PD_{50}) and a half maximal lethal dose (LD_{50}). The paralytic and lethal capacities of the species were calculated as for the nocifensive capacity (see section neuronal cell assays).

(f) Cytotoxicity and membrane potential assays

Drosophila S2 cells (Thermofisher, USA) were maintained and prepared for cytotoxicity and membrane potential variation assays as previously detailed [33,34]. Lyophilized crude venoms were solubilized in ultra-pure water and diluted in culture medium before being exposed to cells at various final concentrations (from 1 ng/mL to 100 µg/mL) for 24 h at 25°C for cytotoxic assays or at 100 µg/mL for 30 min at 25°C for membrane potential monitoring. Cytotoxic assays were performed using lysis buffer and culture medium as positive and negative controls or blanks, respectively. Membrane potential changes were measured using a buffer containing: 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 48 mM sucrose, and 10 mM HEPES. The assays and calculations of LC_{50} were performed as previously described [33].

(g) Phylogenetic analysis

To generate a phylogeny of the studied species we searched for conserved genes across their transcriptome assemblies, or genome for *Pa. clavata*, using BUSCO v5.1.2 [35]. Using the hymenoptera_odb10 database, we identified 566 genes present in at least 90% of the species. We aligned the protein sequences from each gene using mafft and concatenated them into a supermatrix (Dataset S2). Then, we used IQTREE2 v2.1.2 [36] to reconstruct the maximum likelihood tree by using the concatenated matrix and the selection of the best substitution model with ModelFinder and 1000 ultrafast bootstrap replicates. Ancestral states reconstruction for

venom activities and capacities, were estimated by maximum likelihood using the contmap () function of the Phytools R package with default settings [37]. To test for statistical support for correlations between venom bioactivities and morphological traits, and the influences of ecological traits, we used the phylogenetic Generalized Least Squares (PGLS) approach using the PGLS () function of the "caper" package in RStudio. We examined the correlation between each pair of traits individually with the formula set as ([functional traits, e.g. vertebrate_pain] ~ grouping [ecological traits, e.g. defensive_use (yes or no)] and lambda set to "Maximum Likelihood". For the PGLS regressions, we treated log-transformed continuous traits.

3. Results and Discussion

(a) Ecological and venom-related traits

First, we collected observational data about the diet and the use of venom during prey capture or defense to fill the ecological knowledge gap for the studied species. These observations enabled us to define the ecological traits of all the species studied (Figure 1). We did not retain diet specialization as an ecological trait for further analysis since *A. emarginatus* appeared to be an euryphagous predator (i.e. prey on numerous classes of invertebrates) (electronic supplementary material, figure S1) like all other predatory species included in our study, except for *N. commutata*. Additional ecological observations are presented in Figure S13.

We then measured how venom-related traits varied among the 15 ant species (electronic supplementary material, figures S14, S15, and S16). All the morphological data and proportions related to venom yield, venom reservoir volume, sting length, and mandibles length are presented in electronic supplementary material, Table S1 and Table S2. *Pseudomyrmex penetrator* and *Pa. clavata* had the longest stings with ratios of 0.58 and 0.55 and *Odontomachus* spp., *A. emarginatus,* and *P. gracilis* the shortest (ratios ranged from 0.32 to 0.41) (electronic supplementary material, figure S14, E, and Table S1). *Pa. clavata* and *D. armigerum* were the species with the longest mandibles with ratios of 1.0 and 1.1, while *P. penetrator*, *P. viduus* and *P. gracilis* have short mandibles with an average ratio of 0.4 (electronic supplementary material, figure. S14, F and Table S1). We also evaluated the potency of the 15 venoms to trigger nociception in vertebrates and to paralyze and to kill invertebrate prey (electronic supplementary material Tables S3 and S4). The capacity of the venom of a given species is a product of both the venom potency and the amount of venom delivered. To be able to compare species, we therefore calculated both their nocifensive capacity (pain-inducing) and paralytic capacity by dividing the average venom yield (µg) by venom potency (µg/mL) (see details in electronic supplementary material, Tables S3 and S4).

Of the 15 venoms tested with concentrations up to 100 µg/mL, 14 caused an increase in [Ca^{2+}] in the neuronal cell assays (electronic supplementary material, Figure S15), revealing variation in the potency of the different venoms to induce pain (electronic supplementary material,Table S3). The venom of *D. armigerum* was inactive, while that of *A. emarginatus* was active only at the highest concentration tested (100 µg/mL). The estimated median effective concentration (EC_{50}) for the venoms of *D. armigerum*, *A. emarginatus*, *P. viduus,* and *O. scalptus* were estimated to be >100 µg/mL; those of *O. mayi*, *N. commutata*, *O. haematodus*, *O. hastatus,* and *P. gracilis* venoms ranged from 69 (*O. mayi*) to 98 (*P. gracilis*) µg/mL; The venoms of *P. termitarius*, *N. inversa*, *N. goeldii*, *N. apicalis,* and *P. penetrator* were more potent with EC_{50} ranging from 20 (*P. penetrator*) to 53 (*P. termitarius*) µg/mL; The venom of *Pa. clavata* was the most potent of those tested with an EC_{50} of 3.7 µg/mL. *Daceton armigerum*, *A. emarginatus*, and *P. gracilis* have the lowest estimated nocifensive capacities of the species tested; These are followed by *P. viduus*, *O. mayi*, *O. scalptus*, *P. termitarius*, *O. haematodus*, and *P. penetrator*;

The species *O. hastatus*, *N. goeldii*, *N. apicalis*, *N. inversa*, and *N. commutata* have estimated nocifensive capacities that are an order of magnitude greater; finally, *Pa. clavata* has the greatest estimated nocifensive capacity of all of tested species.

All of the tested venoms caused paralysis in blowflies but with different potencies (electronic supplementary material, Figure S16 and Table S4). At 1 h, the venoms of *D. armigerum*, *A. emarginatus*, *P. penetrator*, *P. viduus,* and *P. termitarius* were the most potent paralytic venoms with median paralytic doses (PD_{50}) ranging from 1.2 to 12.9 µg/g, while *Odontomachus* spp. venoms affected the blowflies only at high doses (PD_{50} range from 105.6 to 179.2 µg/g). The venoms of *Neoponera* spp., *P. gracilis,* and *Pa. clavata* had mild paralytic activity (PD_{50} range from 47.4 to 99.6 µg/g). The paralytic effect of *Pa. clavata* venom was reversible and dissipated rapidly, with all blowflies recovering 24 h post-injection except at the highest dose injected (PD_{50}_24h = 265.7 ± 14.5 µg/g), whereas with all other venoms, blowflies remained affected (paralyzed or dead) 24h post-injection. Mild/low lethality was observed, with median lethal doses (LD_{50}) for *Pa. clavata*, *A. emarginatus*, *Neoponera* spp., *Odontomachus* spp., and *P. gracilis* venoms ranging from 245.9 to 677.6 µg/g. By contrast, the venoms of *D. armigerum, P. viduus*, *P. termitarius,* and *P. penetrator* were highly lethal (LD_{50} ranged from 2.0 to 15.5 µg/g). The venom yields allowed us to calculate the paralyzing and lethal capacities of each species. The paralytic capacities at 1 h were particularly high for *P. viduus*, *N. commutata*, and *Pa. clavata* with values of 16.7, 14.6, and 4.6, respectively. These are followed by *P. penetrator*, *D. armigerum*, *N. inversa*, *P. termitarius,* and *A. emarginatus* with values between 1 and 2.3. *Odontomachus* spp., *N. goeldii*, *N. apicalis,* and *P. gracilis* had low paralytic capacities with values <1.

We then evaluated the cytotoxicity of the venoms to gain insight into their mechanism of action. At a concentration of 100 µg/mL, all crude venoms except those of *A. emarginatus* and *D. armigerum* were cytotoxic on *Drosophila* cells (electronic supplementary material, figure S17). The venoms of *Odontomachus* spp. were only cytotoxic at high doses, affecting cell metabolism with LC_{50} values ranging from 15.8 to 42.9 µg/mL and cell membranes with LC_{50} values ranging from 18.5 to 49.0 µg/mL The venoms of *Neoponera* spp. were more cytotoxic with LC_{50} ranging from 5.5 to 8.3 µg/mL and from 5.8 to 14.8 µg/mL for cell metabolism and cell membrane integrity assays, respectively. The *Pseudomyrmex* spp. venoms were very cytotoxic, and the venoms of *P. penetrator* and *P. termitarius* were the most potent, impacting both cell metabolism (LC_{50} of 0.05 and 0.24 µg/mL for *P. penetrator* and *P. termitarius*) and cell membrane integrity (LC_{50} of 0.08 and 0.23 µg/mL for *P. penetrator* and *P. termitarius*). *Pa. clavata* venom was cytotoxic but was more potent on cell metabolism (LC_{50} of 2.72 µg/mL) than on cell membrane integrity (LC_{50} of 31.03 µg/mL). Detailed results are presented in electronic supplementary material, Table S5 and Figure S17. Since no cytotoxic activity was observed for *A. emarginatus* and *D. armigerum* crude venoms, to understand the mode of action we tested the neurotoxic effects of these venoms on *Drosophila* cells. A significant decrease in KCl-induced membrane depolarization was observed after incubation with both venoms, indicating an inhibition effect on ionic conductance (electronic supplementary material, figure S18).

(b) Venom-related traits reveal two functional strategies

A principal component analysis (PCA) was done on the dataset including the venom activities, cytotoxicity, and morphological traits. The first two axes of the PCA accounted for 76% of the total variation, with axes 1 and 2 explaining 44% and 32% of the total variation, respectively (Figure 1A). The vertebrate pain activity and insect cell cytotoxicity have significant loading on PC1 [38] while prey paralysis and sting proportion have significant loading on PC2. PCA revealed that two

different venom strategies are used by the studied ant species. *Anochetus emarginatus* and *D. armigerum* can be defined as species with a non-cytotoxic venom that is capable of paralyzing blowflies efficiently but has a poor capacity to induce pain in vertebrates. All other species are distributed along a venom cytotoxicity gradient, with the most cytotoxic venoms causing more pain in vertebrates, paralysis, and lethality in flies, and tending to have a longer sting.

Among ponerine species, we noted a major shift in insect paralytic activity with *A. emarginatus*, whose venom was 13 to 22 times more paralytic than those of *Odontomachus* species and at least 5 times more paralytic than those of *Neoponera* species (Figure 1B). Reconstruction of the ancestral state of vertebrate pain activity illustrates how the venoms of *D. armigerum* and *A. emarginatus* lack vertebrate pain-inducing ability (e.g. the venom of *A. emarginatus* was 3 to 5 times less active on vertebrate sensory neurons than that of *Odontomachus* species and 100 times less than that of *Pa. clavata*). The amount of venom varied greatly among species, which impacts their paralytic and nocifensive capacities. It is therefore worth noting that the venom of *A. emarginatus* and *D. armigerum* had a very low nocifensive capacity, whereas the venom of *Pa. clavata* and to a lesser extent *N. commutata*, had a high nocifensive capacity (Figure 1B). Altogether, the venom activities and capacities aligned well with the ecological niche of each species. *Anochetus emarginatus* rarely stings defensively, and the sting is not painful, causing only a slight itch (personal observation A.T.) which may explain the cryptic lifestyle of most *Anochetus* species [39]. When disturbed, *A. emarginatus* primarily utilizes its trap-jaw mandibles to bite and bounce off intruders (personal observation A.T. and [40]). *Daceton armigerum* does not sting defensively (personal observation A.T.) and we showed that the crude venom caused no pain-inducing activity. To avoid predation, *D. armigerum* has a very thick cuticle covered with thoracic spines and has adopted an arboreal lifestyle, living in polydomous nests sheltered in hollow branches [41,42]. The defensive constraint against vertebrates in *Pa. clavata* and *N. commutata* may be more pronounced than in other species since they ranked first and second in nocifensive capacity. The large size of workers and the fact that they nest directly in the ground, makes the colony attractive in terms of nutritional resources and highly vulnerable to vertebrate predation. Among ponerine ants, the venom of *Odontomachus* spp. had a low paralytic activity and capacity. *Odontomachus* spp. capture their prey with trap-jaw mandibles and do not always use their venom, depending on the prey type [43,44]. In the *Pseudomyrmex* clade, the venom of the mutualistic plant-ant species (i.e. *P. penetrator* and *P. viduus*) has a very high paralytic capacity on insects, in marked contrast to that of *P. gracilis*. Although *P. viduus* and *P. penetrator* species do not use their venom for predation, they are subject to selective pressures to defend host plants against both grazing insects and vertebrates. *Pseudomyrmex penetrator* venom is also highly effective at inducing pain in vertebrates (electronic supplementary material, Table S3).

(c) Correlation among traits

Comparative phylogenetic generalized least squares (PGLS) regression revealed several significant correlations among traits (Figure 2). For the evolutionary impact of ecological traits, we found that both venom use, and mandible type significantly correlate with venom bioactivities and morphological traits, while there is no correlation between foraging activity (arboreal vs. terrestrial-foraging species) and any other traits (Figure 2A). We show that the metabolic cost of toxin secretion is reduced in stinging ant species that never use their venom for defensive function, since they produce less venom than other ants (proportion of venom reservoir volume ($P = 0.003$)). The defensive function significantly affects the properties of venom: the venoms of species that use their venom defensively generally have greater cytotoxicity

(cytotoxicity_metabolism, P = 0.021; cytotoxicity_membrane, P = 0.031), as well as greater vertebrate pain activity (P = 0.03), associated with higher nocifensive capacity (P = 0.03) than species that use venom exclusively for predatory purposes (i.e. *D. armigerum*, *A. emarginatus*, *P. gracilis*). Counterintuitively, the predatory use of venom significantly reduces the potency against prey, measured as paralysis (P = 0.024) and lethality (P = 0.007) in blowflies, suggesting that some predatory species may compensate low venom activity to capture prey with other adaptations such as trap-jaw mandibles. Mandible strike performances vary among trap-jaw species and may however have a variable influence on the venom activity [48–50]. In this study, the presence of trap-jaw mandibles had no effect on venom activity against blowflies, both paralysis and lethality, but was correlated with low vertebrate pain activity (P = 0.016), low venom volume (P = 0.022), a smaller sting (P = 0.010), low nocifensive capacity (P = 0.021), and low cytotoxicity (cytotoxicity_metabolism, P = 0.007; cytotoxicity_membrane, P = 0.036). The presence of specialized mandibles was therefore associated with an overall decrease in the defensive function of venom.

The data show that the longer the sting, the more pain activity (P = 0.008) and cytotoxicity (cytotoxicity_metabolism, P = 0.004; cytotoxicity_membrane, P = 0.002) were found in the venom, which is consistent with an anti-vertebrate role for a long sting. PLGS regression showed a significant positive correlation between the venom reservoir proportion with both the sting proportion and lethality in blowflies (P = 0.011). Pain activity in vertebrates was strongly positively correlated with the cytotoxicity of venoms (cytotoxicity_metabolism, P < 0.001; cytotoxicity_membrane, P = 0.002) but negatively correlated with the paralysis in blowflies (blowfly_paralysis_1h, P = 0.018; blowfly_paralysis_24h, P = 0.014) (Figure 2B). This is suggestive of a trade-off between vertebrate pain-inducing activity and insect-predation activity in these ant venoms. Such a trade-off might translate to life history strategies, where species with potent paralytic venom against prey have reduced capacity to deter vertebrate predators and are therefore prone to adopt alternative defensive strategies, such as cryptic habits, nesting strategies (e.g. polydomous nest) or promoting behavioral (e.g. thanatosis or escape behavior) and morphological (e.g. thick cuticle and spines; body size reduction) anti-predation co-adaptations.

(d) The venom composition of stinging ants

To understand the biochemical mechanisms underlying the observed variations in venom efficacy, we examined the venom composition of each of the 15 species (Figure 3). Overall, our investigations revealed that the venoms displayed heterogeneity in composition, with a considerable turnover of peptide families across genera and without any correlation with the ecological traits considered (Figure 3). Most of the peptide families were genus specific with only family 9 (ponericin G) shared between *Odontomachus* and *Neoponera* venoms. The venoms of three species *Pa. clavata*, *A. emarginatus*, and *D. armigerum* showed a very distinctive profile dominated by families of neurotoxins not shared with any other venom. Our results also showed that many venom peptide families were shared by species of the same genus, but the proportion varied. For further details on venom composition, see electronic supplementary material, Figures S1-S12 and Dataset S1.

Among the species that use their venom exclusively for predation, *A. emarginatus* and *D. armigerum* were associated with a complete shift in venom bioactivities that correlated with a switch to a neurotoxic venom composition. The venom of *D. armigerum* showed a unique profile, as previously reported [2], and our analysis confirmed that this venom consisted of a single family of peptides (dimeric MYRTX, family 61) that displayed some amino acid sequence similarity with

the neurotoxic U_{11} venom peptide from *Tetramorium bicarinatum* [34] (electronic supplementary material, figure S19). Given the lack of cytotoxicity but strong paralytic activity on blowflies and inhibition of cell membrane potential, these data suggested that *D. armigerum* had an insect neurotoxic venom. In addition, *A. emarginatus* has a non-cytotoxic venom that is much more paralytic to insects than the other ponerine venoms studied. Given the dominance of family 1 peptides in *A. emarginatus* venom (98% of relative expression), it is likely that 2-SS CRPXs (family 1) are responsible for most, if not all, of the paralytic effects upon prey. Since one of the peptides from family 1 (i.e. Ae1a) has shown inhibition (at a high concentration) on the human voltage-gated calcium channel (Ca_V1), it is possible that 2-SS CRPXs are neurotoxins [45]. Although the venoms of *D. armigerum* and *A. emarginatus* shared similar non-cytotoxic, insect neurotoxic activity, the difference in composition and the fact that they were not closely related suggested that they have evolved independently. By contrast, the stings of other ponerine ants *Odontomachus* spp., and *Neoponera* spp. induce sharp pain [46] and showed stronger pain activity in our assay. Ponericins are very prevalent in the venom composition of *Odontomachus*, *Neoponera,* and several other ponerine ants [47–49] but were not present in *A. emarginatus* venom. Ponericins are multifunctional cytotoxic peptides acting on the cell membranes [50], and those from the venoms of *N. apicalis* and *N. commutata* are known to cause pain in mammals and to paralyze insects [46]. There is compelling evidence that membrane-active venom peptides contribute to the defensive role of multiple venomous arthropods against vertebrates [51–53].

Neurotoxic peptides are not exclusive to ants that rely on venom solely for predation. The venom of *Pa. clavata* is largely dominated by poneratoxin (family 62) [18], a pain-inducing neurotoxin that modulates vertebrate voltage-gated sodium (Na_V) channels while paralyzing insects only at very high doses [54]. Despite amino acid sequence similarities with other ant venom peptides [54], its major toxin, poneratoxin (family 62), has never been identified from other ant venoms, including the species studied here. We showed that the venom of *Pa. clavata* also exhibits cytotoxicity, which is likely attributed to phospholipase A_2 (PLA_2), present in this venom at higher levels than in other ants. Since *Pa. clavata* also uses its venom for predation, cytotoxicity may also be a means of subduing arthropod prey. Alternatively, cytotoxicity may also be a means of maintaining a multifunctional defense against predators. In this way, the venom retains a general repellent effect that ensures a baseline defense of the colony in a scenario where a predator would acquire resistance to neurotoxins. This hypothesis is supported by a previous study of the venom of the seed-harvesting ant *Pogonomyrmex*, which uses its venom primarily for defense against vertebrates, and has evolved venoms dominated by vertebrate-selective peptides that target Na_V channels, but still contain a peptide that is cytotoxic to vertebrate cells [55].

Our results indicated that the four *Pseudomyrmex* species have highly cytolytic venoms and different venom profiles to other ant species, with peptide families not shared with the other species studied. We found that *P. gracilis* shares few similarities with the other three *Pseudomyrmex* species, and that the different venom families are present in different proportions among the species. Among these families, family 39 corresponded to the myrmexins (renamed here the dimeric pseudomyrmeciitoxin (PSDTX)), a group of dimeric peptides first described in the venom of *P. triplarinus* [56] which are highly cytotoxic to insect cells [57]. Dimeric PSDTXs largely dominated the venom of *P. viduus* (94% of relative venom expression), which was found to be the most paralytic and lethal venom on blowflies. The other families consisted of cysteine-free polycationic peptides which may also contribute to the observed cytotoxicity by disrupting cell membranes. The venom of *P. penetrator* was at least 3 times more cytotoxic than in other *Pseudomyrmex*, at least 72 times more cytotoxic than *Neoponera*, and at least 231 times more

cytotoxic than *Odontomachus* (electronic supplementary material, Table S5). This was associated with high efficacy for inducing pain in vertebrates and paralyzing insects. This example highlights the fact that the trade-off between vertebrate and insect-predatory venom activity may be disrupted by very high cytotoxicity.

4. Conclusion

Venoms are under strong evolutionary pressures, exerted either on the innovation of toxins or the reduction of the metabolic cost of production. Many ants use venom for subduing a wide range of arthropod prey, as well as for defensive purposes against invertebrates and vertebrates, but are unable to adapt venom composition to stimuli [52,58]. Consequently, the expression of venom genes directly affects the ability of ants to interact with the biotic environment, and the venom composition may be fine-tuned to the ecology of each species. A previous study showed that defensive traits in ants exhibit an evolutionary trade-off in which the presence of a sting is negatively correlated with several other defensive traits, further supporting that trade-offs in defensive traits significantly constrain trait evolution and influence species diversification in ants [17]. However, the sting is not used for the same purpose depending on the ant species. In this study we use a multi-pronged approach to test foraging activity, venom function, and mandible morphology as evolutionary drivers underlying venom variation in ants. We show that ant venoms can be highly heterogeneous and that there have been major shifts in venom composition and bioactivities, even among phylogenetically closely related species. Among the ant species investigated, we found that foraging activity had no impact on venom, but the ecological role of the venom, offensive or defensive, and even more so the breadth of biological targets, were arguably dominant constraints in the evolution of the venom cocktail of these stinging ants. Metering the amount of venom produced is potentially the swiftest way to adapt to different ecological constraints [59] such as for *N. commutata*, which has a quite similar venom composition profile to other congeneric ponerine species but produces large amounts of venom. Evolution may further fine-tune venom composition toward ecologically relevant cocktails exhibiting different functional strategies based on either neurotoxins or cytotoxins. Cytotoxic toxins, which do not require a specific pharmacological receptor, are likely to offer an evolutionary advantage to species that need to target a wide range of both vertebrate and arthropod organisms with highly divergent nervous systems. In some lineages of ants, however, evolution may have favored neurotoxin-based venoms as the range of biological targets narrowed. A reasonable explanation for the prevalence of neurotoxic-based venoms in ants would be that cytotoxic peptides often act at high concentrations compared to neurotoxins [54], and are therefore likely to be associated with higher metabolic costs. Although broader phylogenetic coverage is needed to confirm these findings, our results illustrate the importance of combining functional data with venomic investigations to understand the drivers underlying venom evolution.

Data availability. The raw sequencing reads used in this manuscript are available from the National Center for Biotechnology Information (NCBI) under the project code PRJNA1061791. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD050348.

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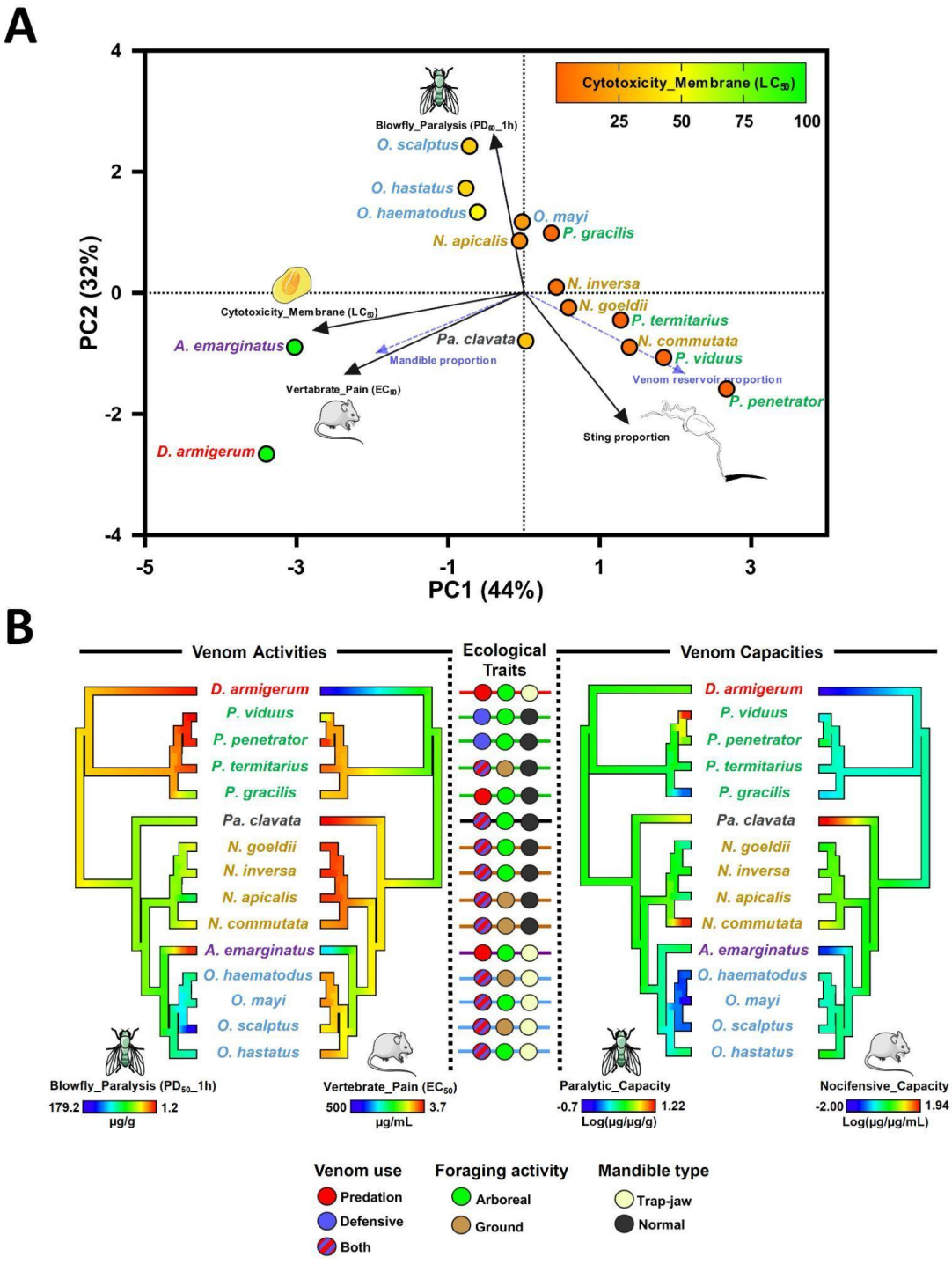
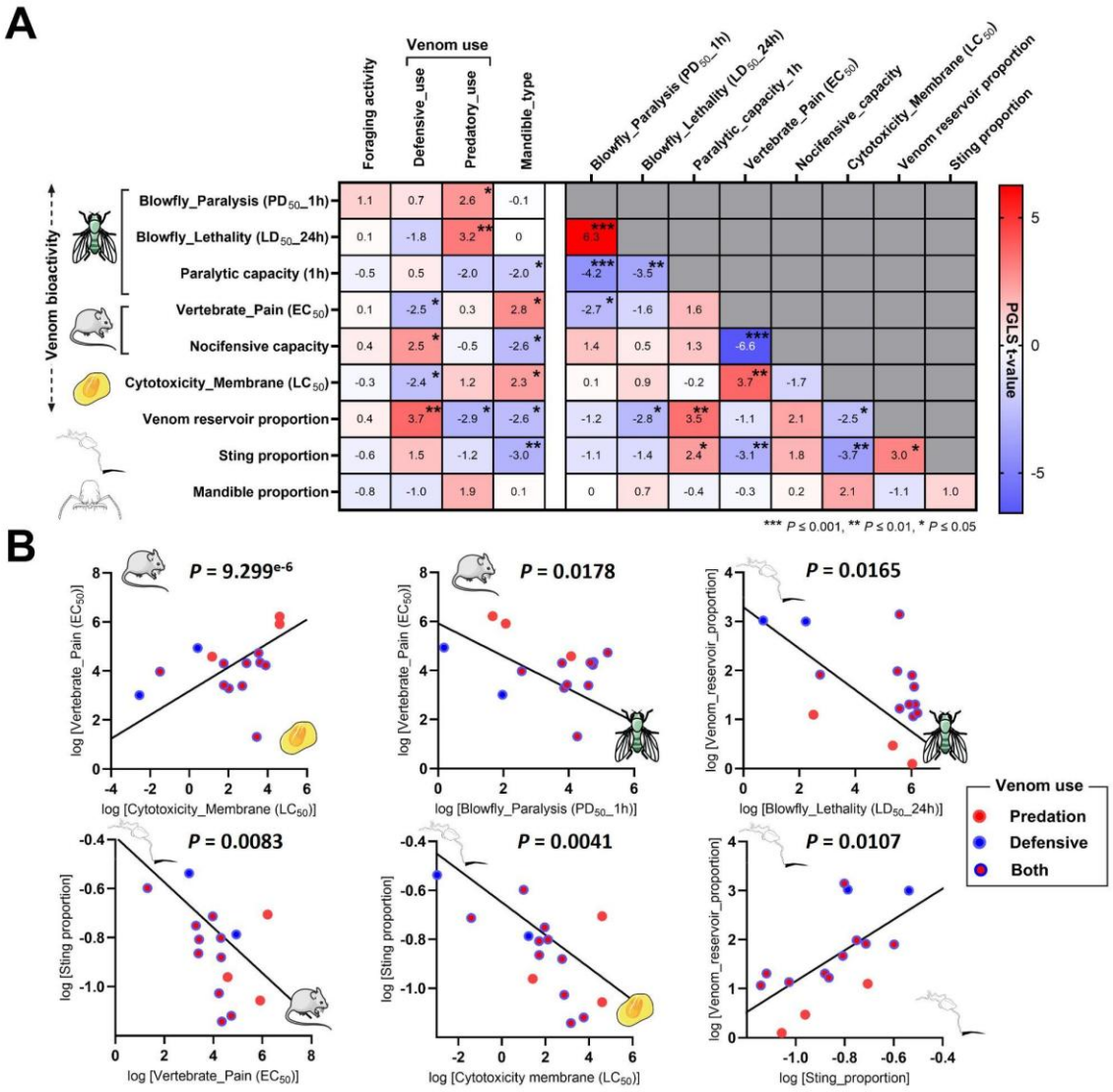
Figures



677

678 **Figure 1.** Venom bioactivity and morphological traits in 15 ant species. A) Principal component
679 analysis of 15 ant species defined by venom bioactivities and morphological features. PCA
680 revealed two functional strategies among species based on the cytotoxicity of venom. The
681 significance of each PC axis, and of loading of each trait have been tested by the PCAtest R
682 package [66]. Traits having significant loadings on PC 1 and PC 2 are represented with black
683 arrows, while others are represented with dashed blue arrows. Plot points are colored in a

gradient based on membrane cytotoxicity values (LD_{50}) as indicated by the scale bar at the top right. See also electronic supplementary materials, figure S7 for PCA on the dataset featuring all traits. B) Ancestral state reconstructions of the insect paralytic activity (PD_{50}_1h), vertebrate pain activity (EC_{50}), paralytic capacity, and nocifensive capacity of crude ant venoms, estimated by using the Phytools R package [67]. Since *D. armigerum* venom was inactive on F11 cells, we used an arbitrary high value of 500 µg/mL for vertebrate pain (EC_{50}). Capacities were calculated by dividing the average venom yield (µg) by the venom potency to paralyze blowfly (PD_{50}_1h, µg/g) and to cause pain (EC_{50}, µg/mL). Venom capacities have been log-transformed. The scale bar indicates trait values from low (cool colors) to high potencies (warm colors) for venom activities and from low (cool colors) to high venom capacities (warm colors). The phylogenetic tree was reconstructed by using transcript sequences of 566 BUSCO genes generated in this study expressed in the body of ant species. Note that the results of the ancestral state reconstruction could be affected by some missing ant lineages in the phylogeny. Species names are colored according to genus (red for *Daceton*, purple for *Anochetus*, blue for *Odontomachus*, brown for *Neoponera*, green for *Pseudomyrmex* and black for *Paraponera*).



699

700 **Figure 2.** Comparative phylogenetic analysis of correlation between traits. A) Phylogenetic
701 generalized least squares (PGLS) analysis among ecological traits, venom bioactivity, and
702 morphological traits in the 15 ant species. As "venom use" is a multi-state discrete variable with
703 non-ordinal properties that contain a category "both", we decomposed that trait into two binary
704 discrete variables (defensive_use and predatory_use) having only two states (yes or no).
705 Heatmap with PGLS t-values and statistical significance. Positively correlated values are in red
706 and negatively correlated values are in blue. B) PGLS linear regressions of several significantly
707 correlated traits.

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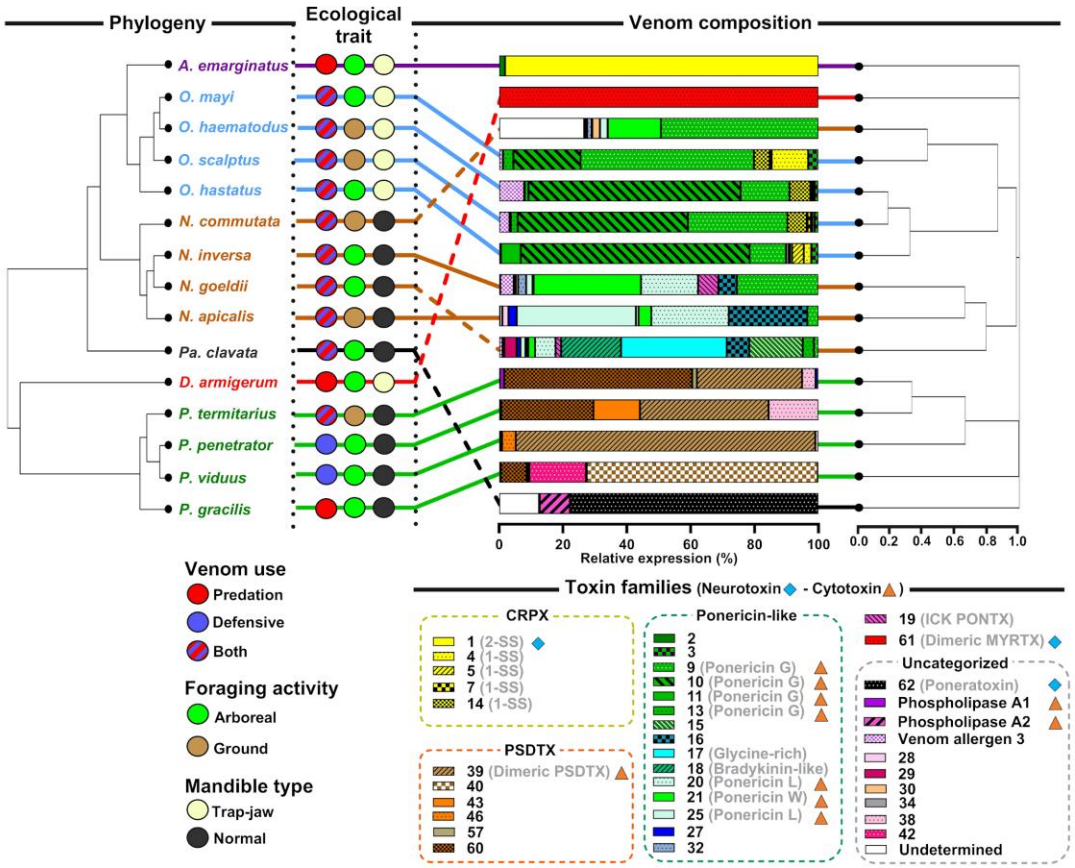

Figure 3. Comparison of venom composition and phylogenetic relationships among 15 ant species. Venom composition cladogram is based on the relative expression (TMM) of transcripts identified as toxins in each venom gland transcriptome and converted into Bray-Curtis distance matrix and hierarchical cluster analysis was performed by using the complete linkage method of hclust() function with the R software. Only families with a relative expression value >1% in at least one species are shown in the color key. Toxin families are grouped by precursor clades (electronic supplementary material, figure S19). Blue diamonds and orange triangles indicate neurotoxic and cytotoxic peptide families, respectively, based on the literature. In contrast to the other species, *A. emarginatus*, *D. armigerum,* and *Pa. clavata* have convergently evolved a venom composition dominated by neurotoxic peptides.