Article - Discovery

# Bee core venom genes predominantly originated before aculeate stingers evolved

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## Keywords

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## Abstract

Venoms have convergently evolved in all major animal lineages and are ideal candidates to unravel the genomic processes underlying convergent trait evolution. However, few animal groups have been studied in detail, and large-scale comparative genomic analyses to address toxin gene evolution are rare. The hyper-diverse hymenopterans are the most speciose group of venomous animals, but the origin of their toxin genes has been largely overlooked. We combined proteo-transcriptomics with comparative genomics compiling an up-to-date list of core bee venom proteins to investigate the origin of 11 venom genes in 30 hymenopteran genomes including two newly sequenced genomes of stingless bees. We found a more distinct pattern in which toxin genes originated predominantly by single gene co-option, a prevalent mechanism in parasitoid wasps. These are always accompanied by parallel expansion events in other bee and hymenopteran sister gene groups. The short toxic peptides melittin and apamin appear to be unique to bees, a result supported by a novel machine learning approach. Based on the syntenic pattern we propose here Anthophilin1 as a bee-unique gene family that includes apamin and MCDP. It appears that bees co-adapted their venom genes which predominantly already existed before the aculeate ovipositor evolved to a stinger which exclusively injects venom. Our results provide insight into the large-scale evolution of bee venom compounds, and we present here the first study revealing general processes of venom evolution at a comparative genomic level for this mega-diverse Hymenoptera.

## Introduction

Molecular processes that drive the evolution of adaptive traits are among the most widely discussed topics in biology (Dunn and Munro 2016; Yin et al. 2016). Venoms, one of the most successful adaptive traits, have arisen by convergent evolution more than 100 times in more than 220,000 species from all major animal groups, covering ~15% of global animal biodiversity (Casewell et al. 2013; Holford et al. 2018; Schendel et al. 2019). Venoms are cocktails of short peptides, enzymes and other proteins that are injected into other organisms for predation, defense or competition, using a specialized morphological structure known as the venom apparatus. The function of venom genes makes them ideal candidates to study the molecular mechanisms that facilitate the evolution of adaptive traits (Drukewitz and von Reumont 2019; Zancolli and Casewell 2020). Advances in comparative genomics and sequencing provide an opportunity to understand those mechanisms at the genomic level (Drukewitz et al. 2019; Zancolli and Casewell 2020; Almeida et al. 2021). Nevertheless, there have been few large comparative studies focusing on the genomic origins of toxin

genes and their weaponization. One reason for this is the predominant interest in venoms for their pharmacological and agrochemical applications or clinical toxinology (Casewell et al. 2013; Holford et al. 2018). Researchers have therefore prioritized groups such as snakes, scorpions, spiders, and cone snails that may not be species-rich compared to insects but are known for powerful venom components of which many cause strong envenomation effects on humans (Holford et al. 2018; Drukewitz and von Reumont 2019). This taxonomic bias hinders a deeper understanding of the origin and evolution of venoms, particularly the vast body of knowledge hidden in the mega-radiations of insects. However, some exceptional insects have been studied in detail recently (for review see Walker et al. 2018; Walker 2020), including the honeybee (Dotimas and Hider 1987; Danneels et al. 2015).

A major dogma in venom evolution is that genes generally gain new functions via duplication followed by neofunctionalization or subfunctionalization (Ohno 1970; Innan and Kondrashov 2010; for the toxinological implications see Jackson and Koludarov 2020). For toxins this process has been illustrated in many cases and species lineages. In toxinology the process of toxin gene origin, referred to as "recruitment", implies that a gene expression location changes from a venom-unrelated tissue to the venom system associated with the acquisition of toxic functions (Nei and Rooney 2005; Casewell et al. 2013; Drukewitz and von Reumont 2019). Genomic research in cnidarians and snakes, the latter is one of the best-studied taxa in this context, suggests that venoms evolve predominantly via gene duplication followed by neofunctionalization and with a subsequent gene loss in several venom protein families (Wong and Belov 2012; Vonk et al. 2013; Moran and Zakon 2014; Dowell et al. 2018; Koludarov et al. 2020; Almeida et al. 2021). However, recent studies (including several in snakes) have challenged this simplistic paradigm and proposed a more complex relationship between gene duplication and neofunctionalization (Dowell et al. 2016; Dowell et al. 2018; Koludarov et al. 2020). Furthermore, gene duplication might not be the driving force behind venom evolution in some vertebrate taxa. In platypus, for example, single gene co-option was proposed as the major recruitment method for toxin genes (Whittington et al. 2018). The same was proposed for parasitoid wasps (*Nasonia* spp.) (Sim and Wheeler 2016; Martinson et al. 2017). Indeed, diverse mechanisms may operate in insects: both gene duplication and single gene co-option were shown to shape the evolution of venom genes in robber flies (Drukewitz and von Reumont 2019).

Hymenopterans (sawflies, wasps, ants and bees) are the most species-rich insect group that are of tremendous ecological and economical importance. They also feature the largest number of venomous species, and close encounters between humans and few common species of pollinating bees or hunting wasps regularly lead to accidental and sometimes lethal envenomation. Unsurprisingly, honeybee and bumblebee venoms are among the best-characterized venoms in the animal kingdom. The venoms of the remaining species of the hymenopteran mega-radiation, including bees with their large number of solitary species, remain largely unexplored despite recent proteo-transcriptomic venom descriptions of several (mostly ant and wasp) species

95 (Moreno and Giralt 2015, **Supplementary Table 1**). However, most of the studies deal with single crude
96 fractions or even individual components either due to technical limitations at the time or because of applied
97 research focus (Lima and Brochetto-Braga 2003; Piek 1986; Lee et al. 2016; Santos-Pinto et al. 2018).
98 Consequently, we still know little about the evolutionary origin and dynamics of hymenopteran and in
99 particular bee venoms, even though high-quality genomic resources are now available for many species (Sim
100 and Wheeler 2016; Branstetter et al. 2018; Elsik et al. 2018).

101 To address this knowledge gap, we combined all relevant verified published toxin data on hymenopterans
102 with new proteo-transcriptomic data of two solitary bees (*Xylocopa violacea* and *Halictus scabiosae*) and the
103 honeybee (*Apis mellifera*) to establish a core venom profile (CVP) for bees (Anthophila). We then used
104 genomic data of 30 species (including two new genomes of stingless bees), representing bees and additional
105 hymenopteran outgroups, for the first taxon-wide comparative genomics analysis to trace the origin and
106 evolution of bee core venom genes. This analysis allowed us to address three key questions: (1) whether bee
107 venoms are dominated by toxin genes that are novel and unique to this clade, (2) whether single gene co-
108 option is the major mechanism of venom gene evolution in bees, as is the case for parasitoid wasps, and (3)
109 whether morphological and ecological adaptations are reflected in the mechanism of venom gene evolution.

## 110 Results

### 111 Venom proteo-transcriptomics

112 To address the lack of detailed bee venom profiles other than for honeybee and bumblebee, we generated
113 high-resolution proteome-transcriptome venom data from two phylogenetically distant solitary bees: the
114 great-banded furrow-bee (*H. scabiosae*) and the violet carpenter bee (*X. violacea*). We also generated
115 complementary data from the honeybee (*A. mellifera*). First, we generated tissue-specific short-read
116 transcriptomes for the whole venom system in all three species. Afterwards we processed the data using a
117 multiple-assembler pipeline (Oyster River Pipeline (MacManes 2018)) to predict open reading frames,
118 allowing us to deduce the translated proteins. Next, we identified secreted venom proteins in the crude venom
119 by Orbitrap-based mass spectrometry (MS) using the venom gland transcriptomes as specific databases. The
120 matched protein sequences were annotated (BlastP searches against UniProt, Uniprot-Apocrita and ToxProt)
121 to identify venom peptides and proteins that are supported by proteome and transcriptome data
122 (**Supplementary Tables 2–4**). We then identified the proteomically supported venom protein families by
123 searching sequences and their annotation against known ToxProt candidates and additionally against
124 described bee specific venom components (**Supplementary Table 1**). We considered transcripts as venom
125 components if they showed a ToxProt bitscore ≥ to the UniProt bitscore to reduce false-positive matches
126 from possible house-keeping genes. In brief, we identified 36 peptide and protein families as venom
127 components in the crude venoms of *A. mellifera*, *H. scabiosae* and *X. violacea.* All three venoms

predominantly contained low-molecular-weight peptides, in particular melittin, apamin and mast-cell degranulating peptide (MCDP). Larger proteins such as phospholipase A2, venom acid phosphatase, venom dipeptidyl peptidase 4 and venom allergens made up less than 10% of the transcriptome (**Figure 1**).

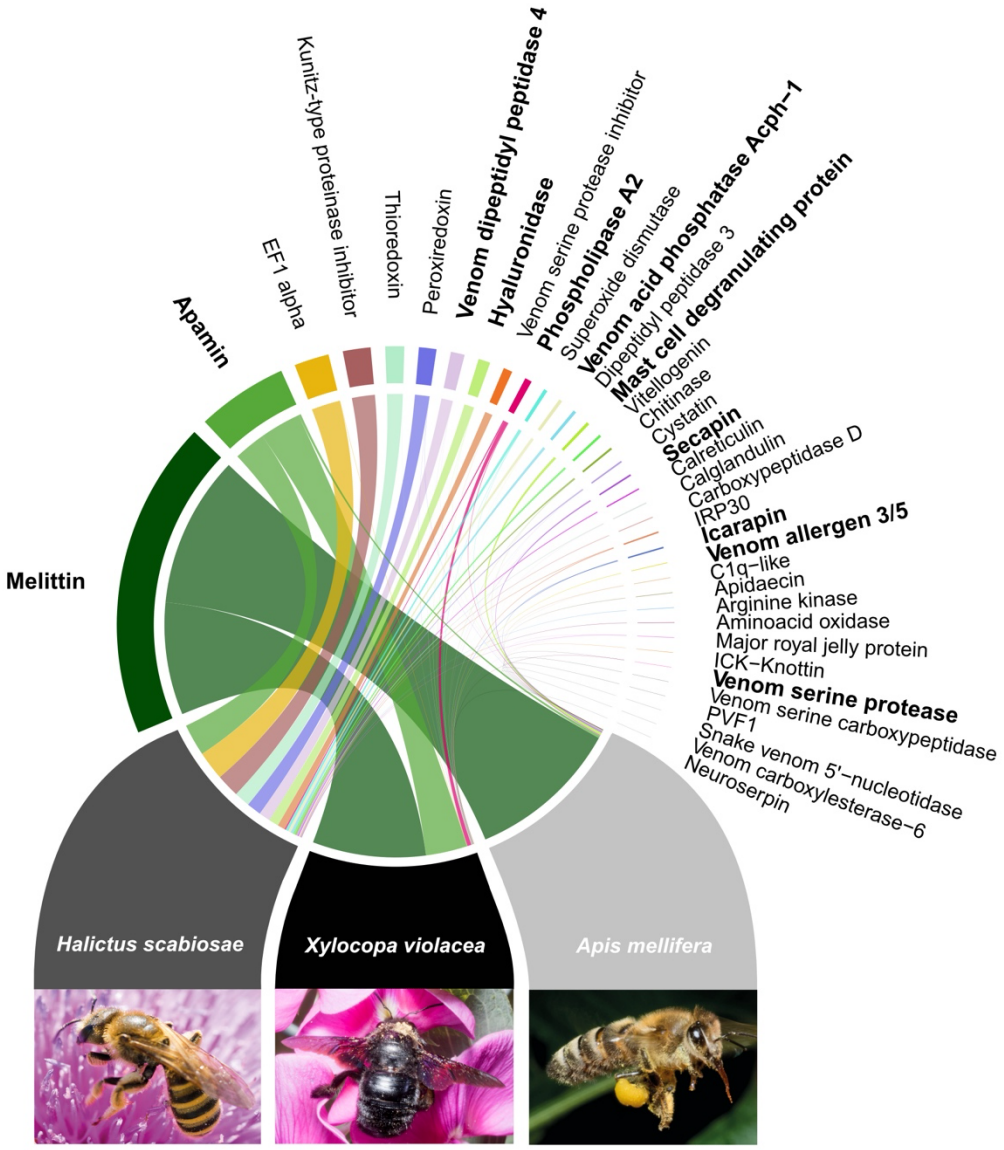



**Figure 1: The venom composition of *A. mellifera*, *H. scabiosae* and *X. violacea*.** Only venom protein transcripts also identified in the proteome are listed here. Transcript expression is shown as a percentage based on scaled transcript per million (TPM) values. The eleven selected venom proteins that we discuss herein further as bee core venom profile are printed in bold.

These results match the few known venom profiles of aculeate wasps and bees, in which small peptides were identified as the dominant components (Lima and Brochetto-Braga 2003; Danneels et al. 2015; Santos-Pinto et al. 2018). Even so, the dominant venom proteins were neither similarly expressed nor necessarily present in all three species. Furthermore, some of the venom components present in the transcriptomes of all three

141 species were only recovered in one of them by MS analysis (Apidaecin, MCDP, and venom allergen isoforms,
142 see **Supplementary Tables 2-4**). One major reason for this very heterogeneous picture is possibly that the
143 physiological state of the venom glands in the used bees could not be synchronized. It was neither possible
144 to keep the solitary bees individuals over several days alive in lab conditions nor possible to establish a
145 milking protocol that the individuals survived. We have to point out that we generated the proteo-
146 transcriptomic datasets in this study predominantly to validate missing venom data for solitary bees to
147 compile a more general core profile of bee venom genes. An in-depth comprehensive discussion of the prote-
148 transcriptomic data will be published elsewhere while we focus here on selected eleven proteo-
149 transcriptomically supported gene families that represent a core venom profile for secreted bee venom
150 components, consistent with the literature on predominant components of injected bee venom (**Figure 2**).

151 **The core venom profile of bees**

152 From our proteo-transcriptomics dataset we selected a set of toxins that we propose as a core venom profile
153 for bees. It is important to note that we focus here on bees as an anchor taxon and venom proteins that were
154 predominantly characterized as the major injected hymenopteran venom components in bees based on known
155 toxic venom components together with literature-based toxin candidates. (Moreno & Giralt, 2015;
156 **Supplementary Table 1**). We restricted our further analysis to these five toxins and six auxiliary venom
157 peptide and protein families mostly with known function, but also including two typical aculeate families
158 with yet unknown biological function (Venom allergen 3/5 and Icarapin). We designated these venom
159 components as a preliminary Core Venom Profile (CVP) in bees, see **Figure 2**. It is important to stress here
160 that we distinguish in the following between toxins with known immediate toxic function such as
161 neurotoxicity (e.g. Apamin) or cytotoxicity (e.g. Mellitin) and venom proteins consistently present in the
162 crude venom but without a direct (or at least known) toxic function.

163 To unravel the evolutionary history of the CVP proteins, we analysed the corresponding genomic regions by
164 searching for homologs in 30 high-quality bee genomes and hymenopteran outgroups (sawflies, jewel wasp,
165 ants, paper wasps), spanning 300 million years of evolution including representatives of the phytophagous
166 sawflies (Symphyta) as oldest hymenopteran group. We first built an exon database of venom genes and their
167 flanking genes based on the well-annotated *A. mellifera* reference genome. We then identified orthologs for
168 each exon in other genomes, which were collected into a secondary, extended database. All genomes were
169 searched using this secondary database and the results were manually inspected to establish completeness
170 and microsynteny, which reflects the arrangement and position of flanking exons of genes around venom
171 protein genes. Our results strongly indicate that venom proteins (as defined above) including enzymatic
172 components are present in highly-stable genomic regions (as multi-copy and single-copy genes). They
173 probably originated in the most recent common ancestor of the earliest diverging hymenopteran lineages

(sawflies) and lineages leading to parasitic wasps and later to derived aculeate wasps. Exceptions from this finding are the short, single-copy genes encoding toxin peptides known from bees such as apamin/MCDP and melittin, which are bee or honeybee unique.

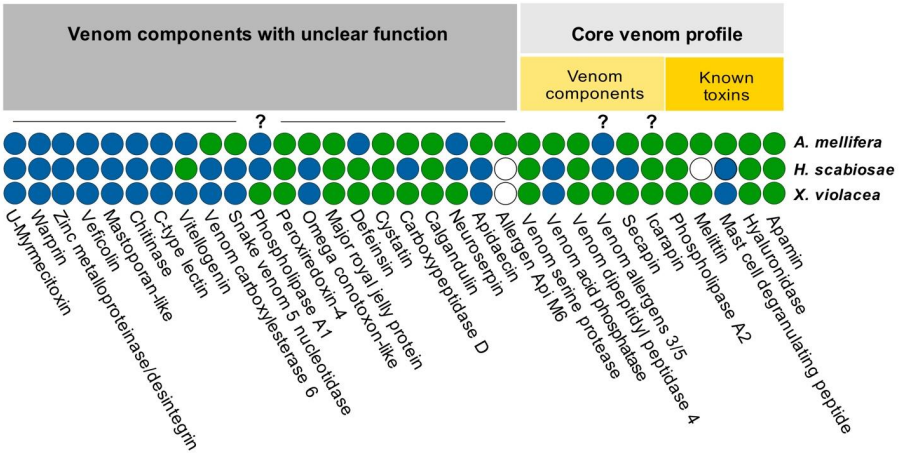



**Figure 2: The core venom profile (CVP) for bees.** Functionally predominantly characterized/injected bee venom toxins and protein families (light grey) and additional venom components with unclear functions (dark grey) were identified in our proteo-transcriptomics dataset and by literature mining. Green circles indicate proteo-transcriptome matches, whereas blue circles indicate transcriptome-only candidates and grey circles indicate the absence of a match. Question marks indicate undefined biological function. See **Supplementary Table 1** for a more detailed overview of known hymenopteran venom components and **Supplementary Table 2-4** for all identified venom components in the present study.

## Apamin is restricted to honeybees and is part of the larger bee unique toxin family Anthophilin1

Apamin, another dominant *A. mellifera* venom component, is encoded by a three-exon gene located next to a very similar three-exon gene encoding MCDP, with which it shares an exon. This tandem duplication is flanked by *MOXD1 homolog 2* and *TBC1 domain family member 30*. Although the two flanking genes are present and identically arranged in the genomes of all the bees we surveyed, we did not detect the full set of apamin or MCDP exons outside of the genus *Apis* (**Figure 3**). Genomic analysis indicated that apamin and MCDP (from *Apis*) are restricted to the Apini clade (*Apis* spp.).

There are, however, multiple uncharacterised genes in Bombini and some other non-*Apis* bees that share the microsyntenic position and basic intron-exon structure with apamin and encode peptides that share cysteine scaffold and signal peptide structure with apamin. Of these apamin-like genes we identified six copies in the *Dufourea* genome, in *Nomia* and *Megachile* five copies, *B. terrestris* two copies, while *Osmia bicornis, Habropoda* and *Megachile* revealed only a single copy.

In addition to that we identified a novel apamin-like gene in *Apis mellifera* located right next to MCDP gene. This gene is potentially a link between *Apis* apamin forms and those of other bees. We identified no similar

genes or exons of apamin in homologous regions from other hymenopterans or in other parts of their genomes.

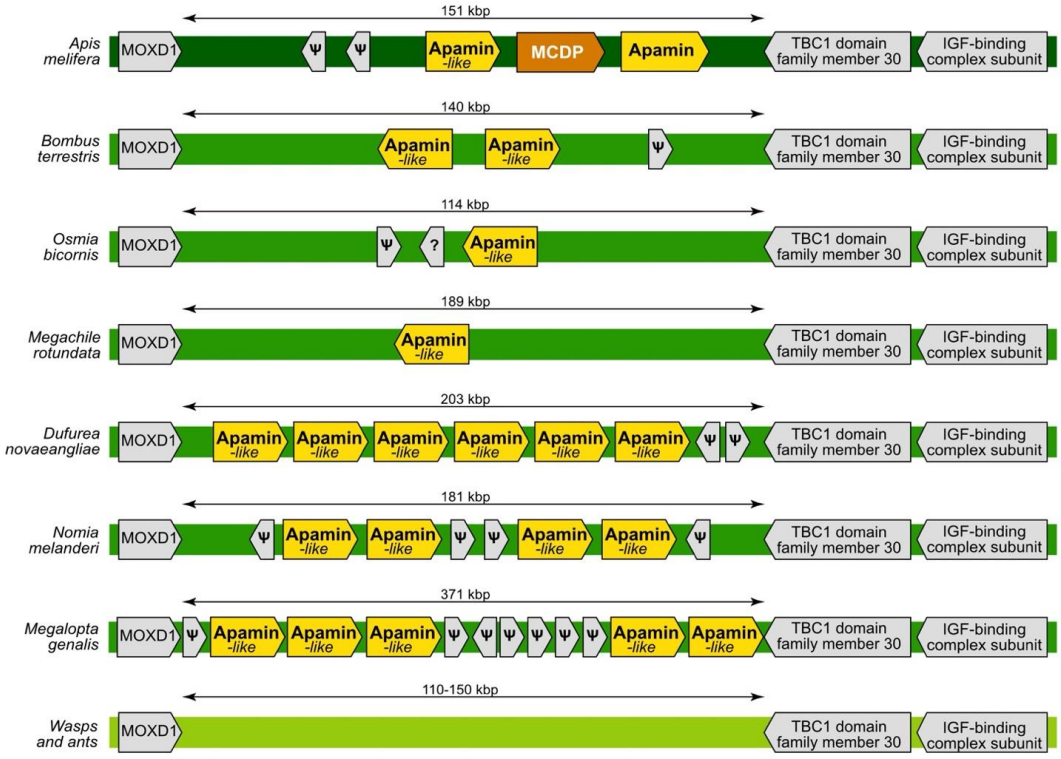


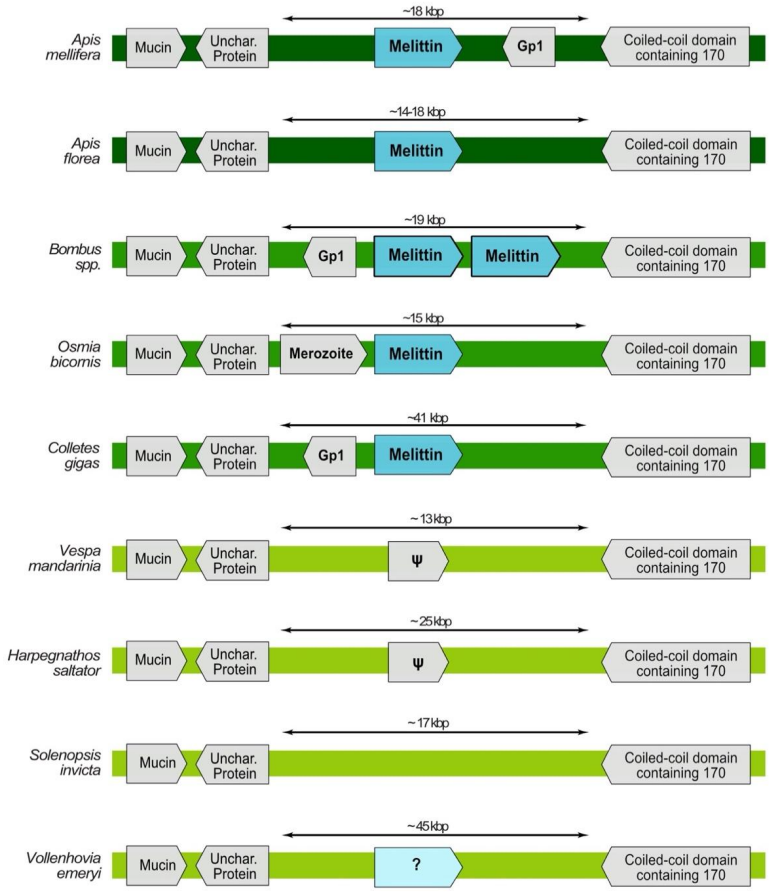

**Figure 3. Gene arrangement and microsynteny for the apamin family (Anthophilin1).** Question marks indicate coding sequences with products of unknown functions. Pseudogenes are symbolized by ψ. The arrows reflect gene orientation. We show here only species for which the genomic sequence in the region with apamin genes is contiguous.

Taken together with the apamin-like sequences we discovered in the core venom profile of *Xylocopa* and *Halictus*, our results indicate that apamin and mast-cell-degranulating peptide are members of a highly variable bee-unique family of apamin-like peptides that undergoes independent duplication events in different lineages. We propose here to rename this family Anthophilin1, which reflects its uniqueness to several lineages within bees (Anthophila), see **Supplementary Figure 1** and **Supplementary File 1** for phylogenetic alignment and tree.

## Melittin is restricted to the bee lineage

Melittin is a key pain-inducing venom peptide in *A. mellifera* (Raghuraman and Chattopadhyay 2007; Chen et al. 2016). The syntenic structure of the *A. mellifera* genome shows that melittin is encoded by a two-exon single-copy gene located between two four-exon genes, one of which encodes *vegetative cell wall protein gp1* while the other remains uncharacterized. Melittin-like sequences in other *Apis* species (*A. dorsata*, *A. cerana* and *A. florea*) feature a similar microsyntenic arrangement (**Figure 4**). Other bee species also possess

218 melittin-like sequences (bombolittin, osmin, collectin, lasioglossin, melectin and macropin (Cerovský et al.
219 2008; Cerovský et al. 2009; Choo, Lee, Yoon, Je, et al. 2010; Stöcklin et al. 2010; Čujová et al. 2014;
220 Monincová et al. 2014; Kawakami et al. 2017). Microsynteny analysis provided evidence that they are
221 orthologous in at least some species from the genera *Colletes, Osmia* and *Bombus* (**Figure 4**).




222

223 **Figure 4: Gene arrangement and microsynteny around the melittin sequence**. All species for which the genome
224 data allowed for a microsyntenic analysis are shown. *Vollenhovia emeryi* was not included in other genomic analyses
225 due to its relatively low genome quality. However, it is shown because it was the only one of the eight analysed ant
226 species that features a seemingly related gene in the correct position but with a very different mature sequence. Genes
227 labelled with ψ in ants and wasps bear little similarity with melittin genes, however, they might be sister genes to the
228 melittin group that underwent severe pseudogenisation.

229

230 In *B. vosnesenski*, the melittin gene has undergone a tandem duplication that is thus far unique to this genus.
231 Some *Bombus* genomes show assembly gaps in this region, preventing the detection of all exons, but recently
232 published genomes of several *Bombus* species (Sun et al. 2021) show the same sequence and duplication in
233 the microsyntenic region identified in *B. vosnesenski* (**Figure 4**). Although tracing the corresponding genomic
234 region in non-bee aculeatans proved to be difficult because of the relative syntenic instability of it, we

successfully located it in ants and wasps with no detectable homology to melittin. However, one particular ant genome – *Vollenhovia emeryi* (excluded from our main genomic analysis due to the relatively low genome contiguity) – had a superficially similar looking peptide in almost the exact location **(Figure 4)**. That peptide has a proline-rich propeptide resembling that of melittin, however, its mature form is very different**.** We conclude here that our results support the hypothesis that melittin is indeed restricted to bee lineages, however, its ancestral gene might have had sister genes in ancestors of wasps and ants, see **Supplementary Figure 2** and **Supplementary File 2** for phylogenetic alignment and tree.

In a previous study it was conjectured that melittin is part of a pan-aculeate family of aculeatoxins that occur in ants, wasps and bees (Robinson et al. 2018). The basis for this grouping is the similarity in signal and propeptides among these proteins, mainly their proline pattern. Other suggested aculeatoxin members include wasp mastoparans and eumenine peptides as well as ant myrmecitotoxins and poneratoxins. Although no experimental or statistical evidence (e.g. model based phylogenetic reconstruction or alignment-based distance matrix) that supports this hypothesis has been published yet, it reflects a plausible evolutionary scenario. For that reason, we test this hypothesis here focused on the evolution of bee venom genes but include representative wasp and ant toxic peptides to trace their possible occurrence in bees: vespid wasps' mastoparan and ants' poneratoxins. We did not find any genomic location and sequences that match eumenine, vespakinins and myrmecitoxin genes in our bee data and thus could not include them.

However, we located poneratoxin-like sequences near or within the *atrial natriuretic peptide-converting enzyme* in all ant genomes of our dataset. The number of the genes seem to vary between the species. Although the genomic region appears unstable, we located atrial natriuretic peptide-converting enzymes and some of its nearby and proximal neighboring genes in *Vespa* and *Apis*. No genomic feature in the genome of *Vespa* seems to relate to poneratoxins, while *Apis* features an uncharacterized small 2 exon gene in a similar position. Aligning it with poneratoxins from *H. saltator*, *S. invicta* and *O. brunneus* (extracted from genomic regions) revealed its close similarity to the 5' end of prepro mRNA of the longest of *H. saltator* poneratoxins, see **Supplementary File 3**. This finding indicates that the original gene got truncated and only the 5' part of it was retained in *Apis*.

We restricted our synteny analysis for mastoparan to the main representative species of bees, ants and wasps and substituted *Polistes* spp. with *Vespa mandarinia* because in both *Polistes* assemblies the mastoparan homologs are located on small scaffolds that prevent the tracing of the synteny. We located mastoparan in the genome of *Vespa mandarinia* and used it as an anchor. The gene is located downstream from *LIM/homeobox protein Lhx9-like*. The genomic region appears stable and we were able to locate it in *Apis mellifera* and *Solenopsis invicta*. However, both species only show annotated pseudogenes (no reading frame seems to resolve in a functional product) in this genomic region roughly in the expected position of

mastoparan. Both pseudogenes have three exons and in both cases one of the reading frames encodes for a proline rich region that could have resembled the mastoparan's propeptide before the pseudogenisation (see relevant part of **Additional File 8).** Given the short peptide sequences and the difficulties to conduct meaningful phylogenetic analyses we tested the acuelatoxin hypothesis focused on melittin further, applying a novel, sequence independent machine learning approach (see methods section for the detail).

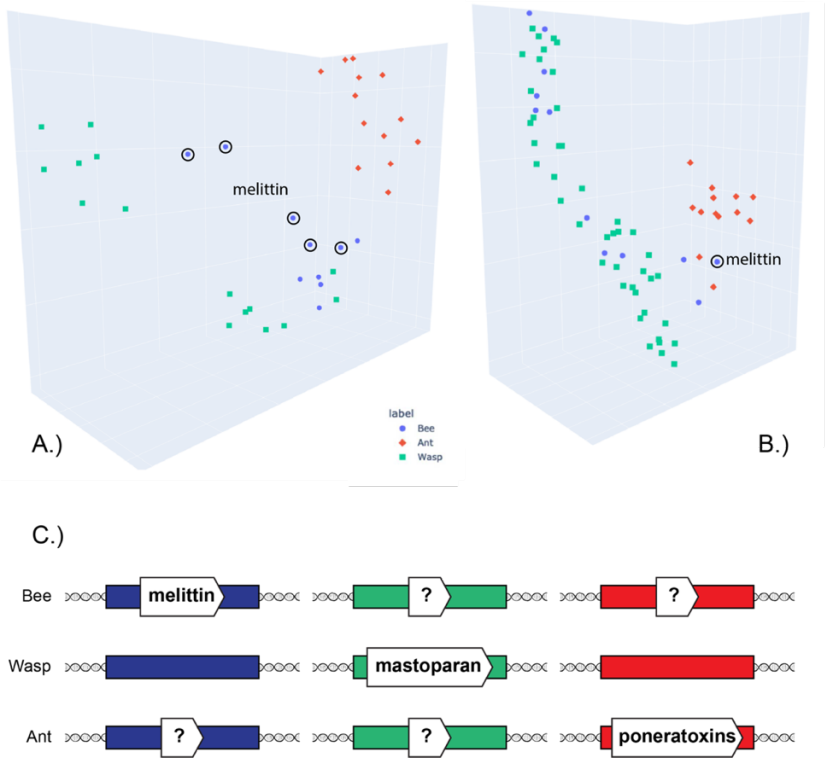



**Figure 5: Structure-function space of small peptidic aculeatan toxins as revealed by the machine learning analysis and their genomic position in respect to each other.** Combined data of available verified toxin sequences from Robinson et al., and the present study. A.) Sequences with signal peptide included B.) Only mature peptides. Melittin sequences are outlined with a black circle, different ones on the panel A represent different transcripts with the same mature peptide. For the interactive plots see **supplementary Files 7 and 8**. C.) Schematic of genomic position of the three groups of hymenopteran toxins. Coloured rectangles represent regions of microsynteny: blue for melittin, green for mastoparan and red for poneratoxins. See text for details.

For the machine learning analyses we used all sequences that Robinson et al presented in their study (kindly provided to us by the author) and all melittin-like toxins known from bees included in our study. We created two datasets – with signal peptides (**Supplementary File 5**, and without signal peptides **Supplementary File 6**), the latter dataset includes more sequences due to the fact that bee melittins are mostly known from proteomic studies and therefore only their mature sequence is known (**Figure 5**). The structure-function space occupied by peptides from wasps and bees revealed in both cases a clear distinction to ants, which is in contradiction to aculeatoxins hypothesis. However, our results reveal a close similarity between bee and wasp

289 peptides, which is even more apparent when signal peptides were removed (which opposes the argumentation
290 of Robinson et al). Our combined results from the microsyntenic analysis and the machine learning approach
291 indeed indicate that melittin is rather independent and unique in bees but convergently similar to genes in
292 wasps and ants.

293 **Abundant venom proteins are encoded by more widespread single-copy genes**

294 Phospholipase A2, hyaluronidase and icarapin are among the most abundant bee venom components (Piek
295 1986; Danneels et al. 2015; de Graaf et al. 2020). Phospholipase and icarapin are encoded by four-exon
296 single-copy genes, whereas the hyaluronidase gene features nine exons. Dipeptidyl peptidase-4 has a strongly
297 conserved single gene, which was present in all hymenopterans in our dataset, probably due to its enzymatic
298 role in the maturation of some toxins. We found that these protein families were highly conserved and
299 ubiquitously present in the genomes of bees, wasps and ants (**Figure 6,** see also **Supplementary Files 8-11**
300 and **Supplementary Figures 3-6** for phylogenetic alignments and trees). Our results support the hypothesis
301 that these genes were recruited into venom functions without any associated duplication – similar to the co-
302 option of single-copy genes proposed as the main process of *Nasonia* venom protein evolution (Martinson et
303 al. 2017). In comparison, phospholipase A2 genes in viperid snakes have undergone significant multiplication
304 and diversification before recruitment into the venom system (Dowell et al. 2018; Koludarov et al. 2020).

305 **Some venom proteins form multi-copy gene families with ancient duplication events**

306 Interestingly, we find larger duplication and diversification events restricted to protein families that comprise
307 enzymatic or larger proteins and not toxin peptides or proteins. Three venom protein classes in the CVP
308 showed copy number variation across the dataset: venom allergens 3/5, venom acid phosphatases, and serine
309 proteases. For these three multi-copy gene families, we found different copy numbers in different species
310 (**Figure 6**). The genes were found in genomic regions that appear evolutionarily stable and allow for the
311 tracing of homologous regions between species by screening for microsynteny.

312 Among the venom acid phosphatases, for which we identified 10 subfamilies (**Figure 6**), the largest
313 expansion of genes occurred in subfamily 7, which is exclusively found in parasitoid wasps. In contrast, the
314 large gene expansion in subfamily 10 appears to be an ancient pattern that is found in sawflies (9 genes) and
315 parasitoid wasps (13 genes). In all remaining hymenopterans, including bees, only one or occasionally two
316 to three genes are present.

317 A similar pattern, though on a smaller scale, was observed for subfamily 5 with ant species having 2-4 copies,
318 while all other hymenopterans (with the exception of *Athalia*) have 1. At the same time subfamily 3 seems
319 to have undergone multiple duplication events in some bee species with up to 10 copies in *Ceratina* and
320 *Bombini*, while other species have 1-2 copies or lost all genes (*meliponini*), see **Supplementary File 12** and

**Supplementary Figure 7** for phylogenetic alignment and tree).

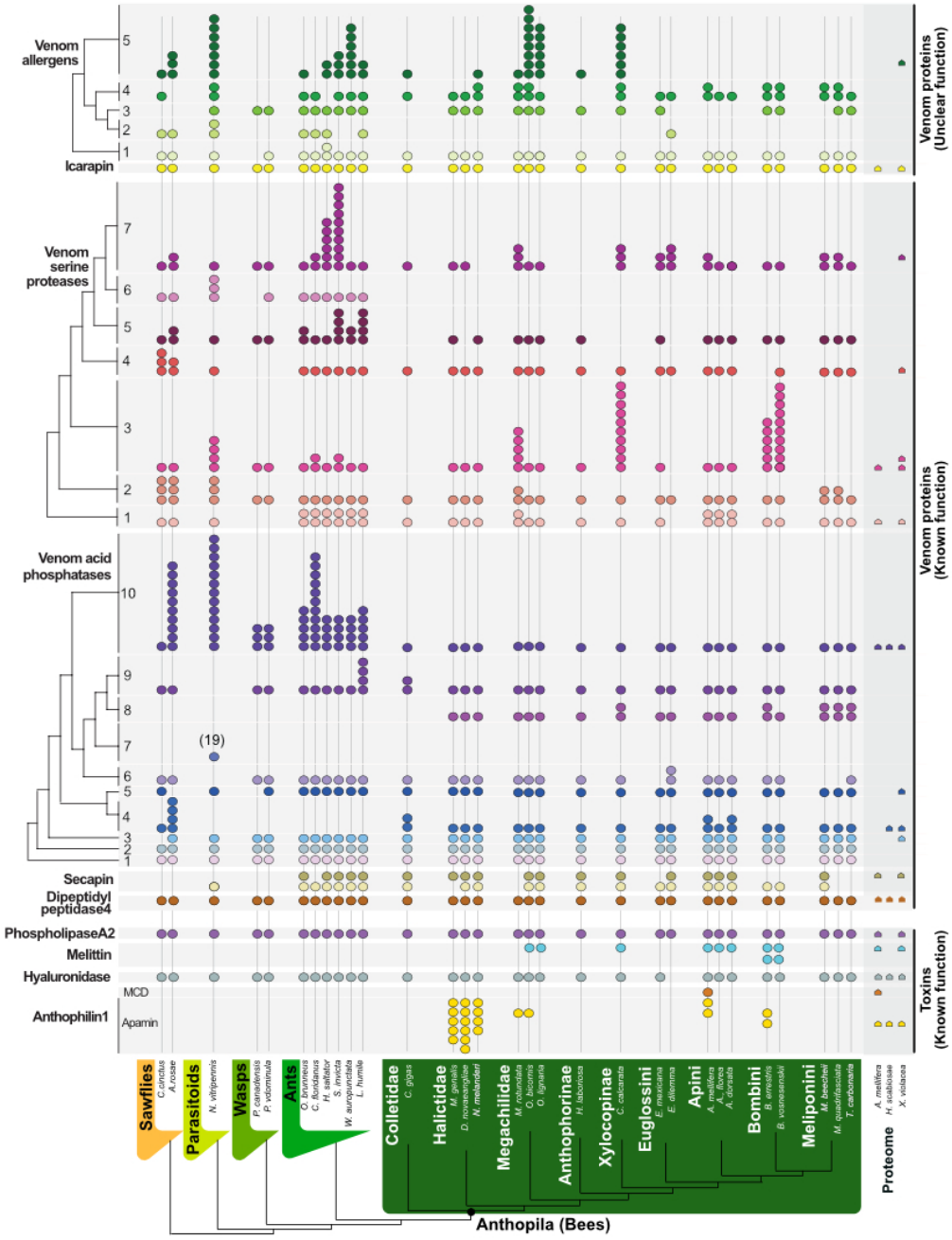



**Figure 6. Overview of all core venom profile (CVP) genes.** The presence of venom gene orthologs and copy number variation is mapped onto the phylogenetic relationship between the species we surveyed (Peters et al. 2017). Coloured circles represent genes with identical microsynteny in the genomes of the surveyed species.

Venom serine proteases are divided into seven subfamilies according to our analyses, among which subfamily 7 is represented by 1–4 genes in all hymenopterans but has expanded in ants (10 genes). All seven subfamilies are present in the older lineages of sawflies and parasitoid wasps, with more diversification in families 2, 3

330 and 4. In bees subfamily 6 appears to have been lost (**Figure 6**; see **Supplementary File 13** and
331 **Supplementary Figure 8** for phylogenetic alignment and tree).

332 Venom allergens 3/5 have been identified in many hymenopterans (Hoffman 2006; Piek 1986) and we
333 inferred the presence of five subfamilies in our study. Subfamily 5 appears to have undergone greater
334 diversification in sawflies, parasitoid wasps, ants and the solitary bees *Ceratina*, *Osmia*. Only a single
335 member of subfamily 5 is present in the solitary bees *Habropoda*, *Colletes* and *Nomia* while it is striking that
336 eusocial wasps and bees of the family Apidae (*Apis*, *Bombus, Melipona*, *Frieseomelitta* and *Eufriesea*) appear
337 to have lost all subfamily 5 genes. In contrast, subfamily 1 is only present in parasitoid wasps and ants with
338 a single gene in *Euglossa*.

339 Other subfamilies generally have a single copy in every species with subfamily 4 occasionally experiencing
340 duplication. In general, the distribution of genes in the venom allergen family is dynamic, but shows some
341 phylogenetic patterns, see **Supplementary File 14** and **Supplementary Figure 9** for phylogenetic alignment
342 and tree). Two secapin genes were present in most of the genomes we surveyed, but were notably absent in
343 sawflies (indicating an origin in the stem Apocrita) and wasps of the genus *Polistes*. This class of peptides
344 displayed intriguing N-terminal sequence variation but strong C-terminal conservation (see **Supplementary**
345 **File 15** and **Supplementary Figure 10** for phylogenetic alignment and tree). The location of both genes was
346 also strongly conserved, with one always present between exons of the neurexin-1 gene and the other located
347 near the carbonic anhydrase-related protein 10. Our inability to locate both genes in some species may reflect
348 technical issues relating to genome quality and/or the more general challenges associated with the location
349 of small and highly-variable genes.

## 350 Discussion

### 351 Gene expansions are restricted to few venom protein families in major taxa

352 Most of the CVP peptides and proteins are encoded as single-copy genes in the bees and other hymenopterans
353 we screened (**Figure 6**). Our data supports the hypothesis that gene duplications are a less dominant
354 evolutionary mechanism in the evolution of hymenopteran venom components. This hypothesis was already
355 proposed for parasitoid wasps (*Nasonia*), in which single gene co-option was identified as the major driver
356 of venom protein evolution (Martinson et al. 2017). However, we have to point out that our results indicate
357 a more distinct pattern in which especially the heavier protein and enzyme components represent the few
358 families of venom proteins in which large gene duplications and expansions have occurred in conserved
359 genomic regions. These expansions are further restricted to particular subfamilies and larger hymenopteran
360 clades (**Figure 6**). The gene duplications and subsequent gene expansions in venom serine proteases, venom
361 allergens and venom acid phosphatases appear to be 'simple' events restricted to the expansion of few genes.

362 This is in stark contrast to other venomous organisms that have been studied more extensively, such as snakes
363 and cone snails, in which venom genes have evolved rapidly by extensive multiplication, expansion and
364 subsequent deletion (Fry et al. 2009; Jackson et al. 2016; Grandal et al. 2021). We have to point out though
365 that this picture is based on our preselected CVP. This includes the most dominant venom components
366 described, however, bee venoms of course contain more venom proteins. Our results indicate that genes of
367 direct toxins and other (assisting) venom proteins evolve differently in bees. This finding should be tested
368 further in the future using extended venom profiles. Complementary activity studies would be important to
369 address the still undefined biological functions of many venom components, which would in turn support the
370 better interpretation of the evolutionary pattern. A good example here would be venom allergens.

371 Venom allergens (3/5) show a more heterogeneous pattern of gene duplications, especially in subfamily 5.
372 This subfamily has expanded in parasitoid wasps, leafcutter bees (Megachilidae) and carpenter bees
373 (Xylocopinae), but has been lost in other Apidae lineages. We can only speculate about the original and actual
374 biological function of venom allergens in general because until today the only activities tested are related to
375 immune responses in mice and humans linked to allergic reactions (Elieh Ali Komi et al. 2018). No study so
376 far has addressed the possible bioactivity linked to the ancestral and venom variant's biological function.
377 However, maybe the strong allergenic activity reflects an original function in sawflies that was linked to the
378 modulation of the immune response of plants, which was later adapted to animal hosts.

379 Genes representing venom acid phosphatase (APH) subfamily 10 have expanded substantially in parasitoid
380 wasps, ants and sawflies, and the 19 genes of venom APH subfamily 7 are unique to parasitoid wasps. In
381 contrast, bees feature only one or occasionally two genes in any APH family. This may signify that ancestral
382 APHs functioned as pre-digestion factors that allowed the offspring of parasitoid wasps to feed more easily
383 on their host (Danneels et al. 2010) . In bees, the retained APHs may be adapted to defensive functions,
384 potentially supported by the origin of APH subfamily 8, which is unique to bees.

385 Venom serine protease (VSP) subfamilies show more phylogenetic variation, with several subfamilies
386 undergoing secondary loss in bees, wasps and ants. Genes representing VSP subfamilies 8 and 9 have
387 expanded in several ants, and partially also in bees without a clear phylogenetic pattern. However, VSP
388 subfamily 5 appears to have expanded in leaf cutter bees, carpenter bees and bumblebees. VSPs are dual
389 function toxins in bees, triggering the phenoloxidase cascade leading to melanization when injected into
390 insects but acting as spreading factors when injected into mammals, similar to snake VSPs with fibrinogen-
391 degrading activity (Choo, Lee, Yoon, Kim, et al. 2010). We hypothesize that the expansion of VSP genes
392 may be linked to this dual function, achieving more effective defense against insects, arthropods and
393 mammals.

394 **Bee-specific toxin genes encoding for short peptides**

395 Bees produce apamin and melittin as dominant venom components, but their genomic origin beyond the
396 honeybee lineage has not been investigated before. One major difference of these toxin peptides to the
397 previously discussed venom components is that the genomic region in which they are encoded appear more
398 dynamic and less conservative. This picture is also reflected by taxon restricted gene duplications. The
399 genomic region containing a tandem repeat of apamin and mast cell degranulating peptide in *Apis* could be
400 identified in other bee genomes based on microsynteny and the characteristic cysteine scaffold. Interestingly,
401 we reveal multiple duplication events that are restricted to single bee lineages. Our conclusion based on this
402 pattern is that apamin and mast cell degranulating peptide are members of a so far unrecognized, highly
403 variable bee-unique peptide family, which we named Anthophilin1. The genes of this family seem to diversify
404 independently in different bee lineages. Whether the duplication events are linked to neofunctionalization or
405 co-option (as dominant venom component) remains to be addressed in future studies. These should include
406 more contiguous genomic data of additional bee lineages. More importantly, however, complementary
407 venom proteomes will be needed to understand in more detail the recruitment and diversification processes
408 of members of this family into bee venom.

409 We identified melittin in a genomic region with conserved synteny in the genera *Apis, Osmia, Ceratina* and
410 *Bombus* (families Megachilidae and Apidae), with a tandem duplication in bumblebees. The synteny
411 confirmed that melittin-like peptides produced by solitary bees are indeed members of the melittin family.
412 Accordingly, melittin is not unique to *Apis* but originated before the divergence of Megachilid and Apid bees.
413 We did not find a syntenic region or sequences similar to melittin in genomes of bees from the families
414 Andrenidae, Halictidae and Colletidae. If melittin evolved in earlier bee lineages and underwent secondary
415 loss in some families remains unclear from our data due to the lack of high-quality genome assemblies for
416 the early-diverging bee lineages. Our data further indicates that the ant *Vollenhovia* features a gene which
417 vaguely could reflect an ancestral version of melittin, however, the mature sequence looks very different.
418 Nevertheless, we have to note that we cannot rule out the possible origin of melittin in earlier aculeatan
419 lineages until a larger taxon sampling for those and earlier bee lineages are available with high-quality proteo-
420 transcriptome-genome data. Nevertheless, our data supports that melittin is co-opted as a single copy gene as
421 one major component in bees. This contrasts with other venomous lineages such as snakes or cnidarians in
422 which major toxin genes are duplicated to become more dominant (Moran et al. 2008; Margres et al. 2017).
423 In future studies this finding should be tested further by analysing more proteo-transcriptomic venom profiles
424 linked to genomic data.

425 **Melittin's origin in the context of the aculeatoxins hypothesis**

426 Hymenopterans are well known for their short toxic venom peptides. Similar to bees with melittin and apamin
427 as short peptides, ants and wasps likewise produce peptidic toxins known as mastoparans, poneratoxins,

428 eumenins, myrmecitotoxins and vespakinins. These were proposed as members of a family of aculeatoxins
429 (Robinson 2018). Testing this hypothesis focused on bees we did not identify any of these peptides directly
430 in clear-cut primary sequence-based genes in bees. However, we located a pseudogenised gene in *Apis* (as
431 bee representative) and *Solenopsis* (as ant representative) in the syntenic region of mastoparan in *V.*
432 *mandarinia*. In wasps no poneratoxin similar genes are identified, but in *Apis* a truncated gene with a
433 poneratoxin similar 5' end is located in a related syntenic region (see relevant genomic annotations in
434 **Additional Files 8**). Based on these results we conclude that our data supports rather a scenario in which
435 ancestors of all of the three toxin families (poneratoxins, mastoparans and melittins) were already present in
436 their modern genomic locations at the very early stages of Aculeatan evolution – latest before the split of
437 vespid wasps from bees and ants. Any duplication of ants' or bees' toxins happened after that independently
438 and likely continues in several lineages. Focused on bee venoms, this implies that melittin is a bee unique
439 toxin. However, our findings do not reject the aculeatoxin hypothesis but reflect a possibly more complex
440 picture in which bee toxins could have evolved independently. It is also plausible that the three groups do not
441 share a common ancestor and have evolved relatively similar propeptide and signal peptide. Their superficial
442 similarity can be explained via shared maturation and expression mechanisms rather than ancestral relation
443 – as was previously proposed (Robinson et al. 2018). The enzymatic cleavage of the mature peptides melittin
444 and apamin by DPP4 is ensured by a single recognition motif. DPP4 cleaves proline-alanine dipeptide –
445 which is exactly the defining feature of 'aculeatoxins' propeptide. An unconstrained BLAST search reveals
446 similarities between individual toxins and binding sites of some of the enzymes (most notably, mastoparan
447 to ATPase).

448 However, our findings could also be in line with the aculeatoxins hypothesis. Further studies using more data
449 could infer that all the three peptide groups share a common ancestral gene at the earlier stages of
450 Hymenopteran evolution. At this stage there's no data that would allow to unequivocally support either of the
451 discussed scenarios. To elucidate this further, the ancestral genes need to be identified and (based on more
452 proteome data) connected with extended genomic data of non-Aculeatan hymenopterans. Nevertheless, there
453 are many other scenarios of origin for these short peptides possible, including a relationship to venom allergen
454 domains (poneratoxins share superficial sequence similarity with some of the groups of those) and *de novo*
455 emergence from non-coding regions. And of course, if melittin is indeed a related gene to either or both
456 mastoparan and poneratoxin (or other small peptides from hymenopteran venoms) it would fall into a general
457 trend of other CVP genes of bees as discussed below.

458 **Most bee core venom proteins originated in early hymenopterans**

459 The pattern that we infer reveals an ancient origin for most of the CVP genes in bees (**Figure 7**). Most
460 subgroups of its major venom protein gene families show a clear-cut orthology with genes already present in

the earliest hymenopteran lineage (sawflies). Female sawflies use their ovipositor to lay eggs in plants but also co-inject proteins that biochemically interfere with the physiology and immune response of plants to ensure the offspring's survival, thus resembling a primitive venom system (Wang et *al.* 2016). The composition of these original hymenopteran venoms has not been studied in detail.

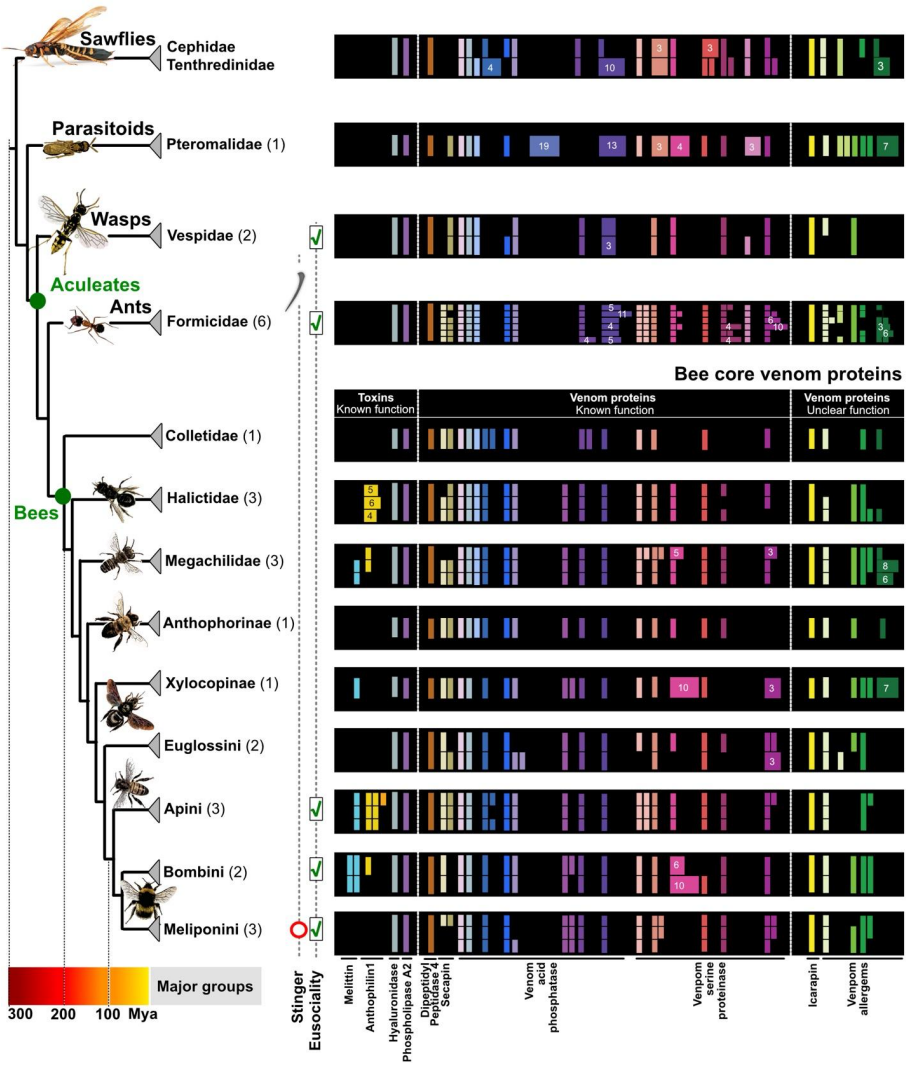



**Figure 7. Simplified visualization of the core venom profile (CVP) proteins of bees and their representation in outgroup taxa.** The numbers of genomes are shown in brackets after the family names**.** Genes are colour-coded and feature a colour range for duplicates. Duplications are summarized by numbers. Phylogeny and divergence times are shown as previously described (Peters et al. 2017).

Our results suggest that the core venom genes present in bees today were already present in the early Triassic in ancestors of the symphytan lineage, predating the radiation of apocritans starting more than 200 million years ago (**Figure 7**) (Peters et al. 2017). The restricted waist of apocritans is needed to manoeuvre the ovipositor in such a way that allows its use for predation, parasitism or defense, and only in aculeate

475 hymenopterans (ants, bees and wasps) is the retractable ovipositor modified into a stinger used exclusively
476 for venom injection. Our data suggest that the genes encoding the predominant bee venom proteins emerged
477 long before the morphological adaptations of a narrow waist and the stinger in aculeates did, which gave this
478 group its common name – the stinging wasps. The core bee venom profile, including known allergens such
479 as phospholipase A2, icarapin and hyaluronidase, was not only already present in sawflies, but is also still
480 present in a group of bees that has secondarily reduced or lost its stinger (stingless bees, Meliponini).

481 One general hypothesis might explain this pattern, namely that hymenopteran venom is a biochemical
482 machine tailored around production and facilitation of small and highly abundant toxic peptides. Thus, neither
483 means of injection (stinger or ovipositor) nor physiology on the receiving end (plant, developing egg or adult
484 animal) would majorly affect it, since any required adaptive change would most dramatically affect the nature
485 of those small abundant peptides as main interactors. Other enzymes would either be unaffected at all (if they
486 are facilitators of the main peptide) or change slightly to adapt to biochemical particularities of a new
487 ecological reality (e.g. serine proteases), varying in their effectiveness but never rendered useless due to
488 broadly applicable mode of action. The subject of this study, bees, seem to support this by having little
489 variation in their venom genes, but for the Anthophilin1 and Melittin groups.

## 490 Conclusion

491 Our comparative analysis provides the first comprehensive insight into evolution and origin of toxin genes
492 in bees. We found that most genes of the predominant bee venom proteins (reflected by the core venom
493 profile) originated at the base of the hymenopteran tree. This means that they already evolved in the last
494 common ancestor of phytophagous sawflies and apocritan Hymenoptera more than 280 million years ago
495 (**Figure 7**). Only the short peptides melittin and the newly described herein family Anthophilin1 that is
496 constituted by apamin, apamin-like and MCDP-like genes are unique to bees. In contrast to other venomous
497 organisms such as snakes, the origin of bee venoms appears to be the result of a process that is based
498 predominantly on single gene co-option, in agreement with previous findings in parasitoid wasps. Gene
499 duplications occur, but only in certain (not major toxin) protein families and in only a few hymenopteran
500 lineages, reflecting a generally more diverse pattern of gene origin. As a consequence, our results indicate
501 that toxin and venom protein genes probably evolve under different evolutionary processes. Our results
502 (limited to the core venom profile) establish requirements for future studies to provide insight into the
503 evolution of bee and hymenopteran venoms. These should include more high-quality genomes, especially for
504 early bee lineages but also including a more widespread taxon sampling of other hymenopterans. More
505 importantly, extended proteo-transcriptomics venom profile data are essential. Corresponding to genomes
506 this data improves genome annotations and allows to address appropriately venom protein recruitment
507 processes to differentiate more precisely between gene variants expressed in the venom system and non-

508 venom related genes. Finally, bioassays for many still unknown venom components are needed to identify
509 functional differences linked to the gene evolution and diverse ecology of hymenopteran species.

## 510 Materials and methods

511 **Sample collection and preparation.** All specimens of *X. violacea* (15 individuals) and *H. scabiosae* (17
512 individuals) were collected in June and July 2019/2020 in the alluvial area of the River Wieseck in Giessen,
513 Germany, with permission from the HNLUG Giessen (IV.2 R28). *A. mellifera* specimens (15 individuals)
514 were obtained from the beehive at the Institute for Insect Biotechnology at Justus-Liebig-University Giessen.
515 The whole venom system of each specimen was dissected on ice under sterile conditions. We extracted crude
516 venom from glands and venom reservoirs by squeezing with forceps in sterile ultrapure water (Thermo Fisher
517 Scientific, Waltham, MA, USA) after prewashing twice to minimize hemolymph contamination. The
518 remaining tissue was preserved in RNAlater (Thermo Fisher Scientific) for subsequent RNA sequencing.

519 **RNA extraction and venom gland transcriptome sequencing.** RNA extraction, library preparation and
520 short-read genome sequencing were outsourced to Macrogen (Seoul, Korea) for *A. mellifera* and *X. violacea*e
521 and to Novogene (Cambridge, UK) for *H. scabiosae*. RNA was extracted with Trizol and after a successful
522 RNASeq quality control the cDNA libraries (150bp, paired end reads) were sequenced using a low input
523 protocol (Illumina Truseq) on an Illumina HiSeq2500 (Macrogen) and Illumina NovaSeq (Novogene). For
524 *H. scabiosae* an in-house ultra-low input protocol was used by Novogene due to very low RNA concentration
525 and quantity. All raw data are submitted to NCBI GenBank PRJNA733472 (SRA entries: SRR14690757,
526 SRR14690758, SRR14690759).

527 **Transcriptome assembly, protein prediction and annotation.** Venom gland transcriptomes were
528 assembled separately using Oyster River Pipeline v2.2.6 (MacManes 2018) with TPM filtration set to 1. The
529 resulting assemblies were processed using Transdecoder (minimum length 20 amino acids) to predict
530 peptides, and Kallisto v0.46 (Bray et al. 2016) to calculate individual transcript abundance, see **Additional**
531 **Files 1-3**. The assembled transcripts and their corresponding longest ORFs (Transdecoder output) were used
532 as local BLAST queries against ToxProt and UniProt (the latter limited to insects only) with an e-value cutoff
533 of $1 \times 10^{-3}$. Any highly abundant (TPM > 100) transcripts without significant matches were manually
534 screened using BLAST, InterPro scan and ProteinPredict online suites to determine the closest characterized
535 homolog. For subsequent venom protein identification, we only included transcripts identified in our
536 proteomic dataset representing proteins secreted in the venom system. To compare subsequently all venom
537 proteins in the three datasets we calculated the percentage of scaled TPMs using the package txtimport on R,
538 the script is available via github (https://github.com/marivelasque/VenomEvolution.git), see **Supplementary**
539 **Tables 2-4**. Relatively low BUSCO value for *H. scabiosae* transcriptome could be explained by the fact that
540 of the three species its glands are by far the smallest and thus gland-specific transcripts will have higher

541 abundance compared to housekeeping transcripts, as well as computed score being lower than actual due to
542 BUSCO methodology inflating the proportion of missing genes as elaborated by Simão et al (Simão et al.
543 2015). Due to the protection status of bees and subsequent nature conservation regulations we were limited
544 in the number of specimens and could not dramatically increase the sample size to increase tissue quantities.

545 **Table 1: Overview of the newly-generated transcriptome and proteome datasets for the venom glands**
546 **of *A. mellilfera*, *H. scabiosae* and *X. violacea*.**

| | *A. mellifera* | *H. scabiosae* | *X. violacea* |
|---|---|---|---|
| Illumina reads (paired end, 150 bp) | 26,092,431 | 38,605,338 | 16,077,242 |
| Assembled transcripts | 31,282 | 80,388 | 63,006 |
| BUSCO values | 97.7% | 41.6% | 97.4% |
| Predicted peptides | 74,609 | 94,492 | 144,998 |
| MS-supported protein matches | 121 | 211 | 187 |

547 **Proteomic analysis of crude venom.** All transcriptome assembly-based predicted ORFs were used as
548 specific databases to identify peptides and proteins detected by mass spectrometry. For the tryptic digestion
549 of the crude venom from *H. scabiosae*, we dissolved 10 µg of protein in 10 µl 10 M urea containing 0.1%
550 ProteasMax (Promega. Madison, WI, USA). Cysteine residues were reduced with 5 mM DTT (30 min at 50
551 °C) and modified with 10 mM iodoacetamide (30 min at 24 °C). The reaction was quenched with an excess
552 of cysteine and trypsin was added at a protein:enzyme ratio of 40:1 in 100 µl 25 mM ammonium bicarbonate
553 (Sigma-Aldrich, Taufkirchen, Germany). After incubation for 16 h at 37 °C, the reaction was stopped by
554 adding 1% trifluoroacetic acid (TFA). The sample was purified using a C18-ZipTip (Merck-Millipore,
555 Darmstadt, Germany), dried under vacuum and redissolved in 10 µl 0.1% TFA.

556 LC-ESI-MS analysis was carried out at 35 °C by loading 1 µg of the sample in 0.1% formic acid (Sigma-
557 Aldrich) onto a 50-cm µPAC C18 column (Pharma Fluidics, Gent, Belgium) mounted on an UltiMate
558 3000RSLCnano (Thermo Fisher Scientific). Peptides were eluted with a linear gradient of 3–44% acetonitrile
559 over 240 min followed by washing with 72% acetonitrile at a constant flow rate of 300 nl/min. They were
560 then infused via an Advion TriVersa NanoMate (Advion BioSciences, New York, NY, USA) into an Orbitrap
561 Eclipse Tribrid mass spectrometer (Thermo Fisher Scientific) in positive-ionization mode with a NanoMate
562 spray voltage of 1.6 kV and a source temperature of 275 °C. Using data-dependent acquisition mode, the
563 instrument performed full MS scans every 3 s over a mass range of *m/z* 375–1500, with the resolution of the
564 Orbitrap set to 120,000. The RF lens was set to 30%, and auto gain control (AGC) was set to standard with a
565 maximum injection time of 50 ms. In each cycle, the most intense ions (charge states 2–7) above a threshold
566 ion count of 50,000 were selected with an isolation window of 1.6 *m/z* for higher-energy C-trap dissociation
567 at a normalized collision energy of 30%. Fragment ion spectra were acquired in the linear ion trap with the
568 scan rate set to rapid, the mass range to normal and a maximum injection time of 100 ms. After fragmentation,
569 the selected precursor ions were excluded for 15 s for further fragmentation. The raw proteomics data have

570 been deposited to PRIDE with the dataset identifier PXD026642.

571 Prior to shotgun proteomics, the *X. violacea* and *A. mellifera* venom samples were denatured, reduced, and
572 alkylated. Briefly, each sample (~50 µg) was dissolved in 89 µl 100 mM triethylammonium bicarbonate
573 (TEABC), and cysteine residues were reduced by adding 1 µl 1 M DTT (30 min at 60 °C) and modified by
574 adding 10 µl 0.5 M iodoacetamide (incubation for 30 min in the dark). We then added 2 µg trypsin (Promega)
575 in 100 mM TEABC and incubated overnight at 30 °C. The peptides were then purified and concentrated
576 using OMIX Tips $C_{18}$ reversed-phase resin (Agilent Technologies, Santa Clara, CA, USA). The peptides were
577 dehydrated in a vacuum centrifuge and analysed by NanoLC-MS/MS. Briefly, the samples were resuspended
578 in 20 µl buffer A (0.1% formic acid) and 1 µl was loaded onto an analytical 25 cm reversed-phase column
579 (Acclaim Pepmap 100 $C_{18}$) with a 75 mm inner diameter (Thermo Fisher Scientific) and separated on the
580 Ultimate 3000 RSLC system coupled via a nano-electrospray source to a Q Exactive HF-X mass spectrometer
581 (Thermo Fisher Scientific). Peptides were separated using a 6–40% gradient of buffer B (80% acetonitrile in
582 0.1% formic acid) over 123 min at a flow rate of 300 nl/min. Using data-dependent acquisition mode, full
583 MS/MS scans (375–1500 m/z) were performed in the Orbitrap mass analyser (Thermo Fisher Scientific) with
584 a 60,000 resolution at 200 m/z. For the full scans, 3 × 106 ions accumulated within a maximum injection time
585 of 60 ms. The 12 most intense ions with charge states ≥ 2 were sequentially isolated to a target value of 1 ×
586 105 with a maximum injection time of 45 ms and were fragmented by higher-energy collisional dissociation
587 in the collision cell (normalized collision energy 28%) and detected in the Orbitrap mass analyser at a
588 resolution of 30,000. PEAKS Studio v8.5 (Bioinformatics Solutions, Waterloo, ON, Canada) was used to
589 match MS/MS spectra from X. violacea and A. mellifera venom samples against an in-house database
590 resulting from the annotated transcriptome of each species. Carbamidomethylation was set as a fixed
591 modification, and oxidation of methionine as a variable modification, with a maximum of three missed
592 cleavages for trypsin digestion. Parent and fragment mass error tolerances were set at 5 ppm and 0.015 Da,
593 respectively. A false discovery rate (FDR) of 1% and a unique peptide number ≥ 2 were used to filter out
594 inaccurate proteins. A −10lgP value > 120 was used to estimate whether detected proteins were identified by
595 a sufficient number of reliable peptides. In order to identify more relevant sequences, the Spider algorithm
596 (PEAKS Studio) was used to find additional mutations or to correct sequences. This algorithm corrects the
597 sequences stored in transcriptomic databases with de novo sequences based on MS/MS spectra, allowing the
598 detection of post-translational modifications (PTMs) and mutations. The minimum ion intensity for PTMs
599 and mutations was set to 5%, and the ALC score was set to ≥ 90 for de novo sequences, leading to low
600 precursor mass errors. Transcripts supported by proteomic data were manually filtered by excluding non-
601 venom-related proteins and peptides, such as house-keeping and structural genes (**Supplementary Tables 2-**
602 **4**). Proteome raw data are submitted to PRIDE (PXD029934, PXD029823).

603 **Genome mining, sequencing and annotation.** For our comparative analysis of venom toxin proteins across
604 the order Hymenoptera, we made use of the following publicly available genome sequences: *Apis cerana*
605 (GCA001442555.1), *Apis* dorsata (GCA000469605.1), *Apis florea* (GCA000184785.2), *Apis mellifera*
606 (GCA003254395.2), *Athalia rosae* (GCA000344095.2), *Bombus terrestris* (GCA000214255.1) *Bombus*
607 *vosnesenskii* (GCA011952255.1), *Camponotus floridanus* (GCA003227725.1), *Cephus cinctus*
608 (GCA000341935.1), *Ceratina calcarata* (GCA001652005.1), *Colletes gigas* (GCA013123115.1), *Dufourea*
609 *novaeangliae (GCA001272555.1), Eufriesea mexicana* (GCA001483705.1), *Euglossa dilemma*
610 (GCA002201625.1), *Friseomelitta varia* (GCA011392965.1), *Habropoda laboriosa (GCA001263275.1),*
611 *Harpegnathos saltator* (GCA003227715.1), *Linepithema humile* (GCA000217595.1), *Megachile rotundata*
612 (GCA000220905.1), *Megalopta genalis* (GCA011865705.1), *Melipona quadrifasciata* (GCA001276565.1)
613 *Nasonia vitripennis* (GCA009193385.2), *Nomia melanderi* (GCA003710045.1), *Odontomachus brunneus*
614 (GCA010583005.1), *Ooceraea biroi* (GCA003672135.1), *Osmia bicornis* (GCA004153925.1), *Osmia*
615 *lignaria* (GCA012274295.1), *Polistes canadensis* (GCA_001313835.1), *Polistes dominula*
616 (GCA001465965.1), *Solenopsis invicta* (GCA_016802725.1), *Vespa mandarinia* (GCA014083535.1),
617 *Vollenhovia emeryi* (GCA000949405.1), *Wasmannia auropunctata* (GCA000956235.1) (Kapheim et al.
618 2015; Branstetter et al. 2018; Wallberg et al. 2019; Kapheim et al. 2020). The genomes and annotations of
619 the stingless bees *Tetragobula carbonaria* and *Melipona beecheii* will be published as part of another study,
620 but have already been uploaded to NCBI.

621 We also annotated protein-coding genes based on the genome sequence assembly of *C. gigas*
622 (GCA013123115.1, ASM1312311v1,(Brand et al. 2017) because the annotation presented in the publication
623 is not publicly available. Repeats were soft-masked using RepeatMasker annotations
624 (GCA013123115.1_ASM1312311v1_rm.out) with tabtk, bioawk and seqtk (https://github.com/lh3). We
625 used Funannotate v1.8.1 (Palmer and Stajich 2020) and Uniprot (sprot) for homology-based evidence based
626 on protein sequences from 11 related bee species: *B. impatiens*: GCF000188095.2, *B. terrestris*:
627 GCF000214255.1, *A. mellifera*: GCF003254395.2, *M. quadrifasciata*: GCA001276565.1, *E. mexicana*:
628 GCF001483705.1, *F. varia* GCA011392965.1, *M. rotundata* GCF000220905.1, *H. laboriosa*
629 GCF001263275.1, *D. novaeangliae* GCF001272555.1, *M. genalis* GCF011865705.1, *N. melanderi*
630 GCF003710045.1 (Kapheim et al. 2015; Wallberg et al. 2019). Briefly, funannotate used gene predictions
631 from Genemark-ES, Snap v2006-07-28, glimmerHmm v3.0.4, Augustus v.3.3.3, and CodingQuarry v2.0
632 together with protein alignments in Evidence Modeler v.1.1.1. Too short, gap-spanning or repeat-overlapping
633 gene models were removed (n = 5446) and tRNA genes were detected (n = 168) with tRNAscan-SE v2.0.6.
634 Genes were functionally annotated using PFAM v33.1, the UniProt database v2018_11, EggNog
635 (eggnog_4.5/hmmdb databases: Arthropoda, Insecta, Hymenoptera, Drosophila), MEROPS v12.0,
636 CAZYmes in dbCAN v7.0, BUSCO Hymenoptera models v3.0.2, Hymenoptera odb9, SignalP v4.1, and

637 InterProScan5 v81.0. The final annotation contained models for 20,016 protein-coding genes and 168 tRNAs,
638 and was estimated to be 87.1% complete (BUSCO4 v4.1.4). The resulting gene annotation files for *C. gigas*,
639 *E. dilemma*, *M. beecheii* and *T. carbonaria* will be made available as **Additional Files 4-7** in the Zenodo
640 archive accompanying this manuscript (10.5281/zenodo.5734574).

641 **Genomic analysis.** We traced abundant venom gland transcripts that potentially encoded toxins to homologs
642 in the annotated, highly-continuous publicly-available genomes of bees (and wasps, ants, parasitoid wasps
643 and sawflies as outgroup species) using the online BLAST (Altschul et al. 1990) suite against genomic
644 databases. To identify conserved synteny blocks, we first identified the reciprocal best-match paralogs from
645 hymenopteran all-against-all BLASTP comparisons of the venom genes. Based on the matching sequences,
646 we then extracted exons from the candidate venom genes and their flanking genes. We used those to create
647 BLAST databases to survey the selected genomes using tblastx with an e-value cutoff of 0.01. We then
648 applied filters to select venom genes at least 20 kbp in length (to exclude partial genes) with at least two
649 exons. Where gene annotations were insufficient, we manually re-annotated venom genes where possible,
650 following intron boundaries and using known sequences as templates. We extracted the coding sequences of
651 all complete genes for phylogenetic analysis to establish ortholog groups in addition to their microsyntenic
652 patterns. All resulting annotations are available as part of the Additional Materials (**Additional File 8).**

653 **Phylogenetic analysis.** All toxin transcripts together with toxin genes and their outgroup venom-unrelated
654 homologs (e.g. trypsins and chymotrypsins in case of serine proteases) were arranged by gene family and
655 aligned as translated amino acids using MAFFT (Katoh and Standley 2013) (L-INS-I, 1000 iterations). Name
656 convention was established to differentiate between genomic sequences (first two letters of both genus and
657 species name, followed by the last three digits of a bioinformatic scaffold ID, followed – if applicable - by
658 an abbreviation of a pre-existing gene annotation, followed by letters a to z to differentiate between sequences
659 from the same scaffold); proteo-transcriptomic sequences (names kept the same as generated by
660 transcriptome assemblers); homologues from UniProt and SwissProt databases used to provide outgroups
661 and fill the gaps in sequence space (kept as UniProt or SwissProt IDs). Alignments were manually inspected
662 for overt errors (e.g., proper alignment of the cysteine backbone) and used to construct phylogenetic trees in
663 Exabayes (Aberer et al. 2014) (four parallel runs of four chains each, and 1,000,000 generations). Resulting
664 trees are shown in the **Supplementary Figures 1-10**, with toxin sequences recovered from *Apis, Halictus* or
665 *Xylocopa* venom marked as red arrows and non-toxic physiological sequences marked with grey arrow.

666 **A novel perspective on relations of short peptide: embedding space analysis.** Every year, algorithms
667 improve natural language processing (NLP) tasks such as automated translation or question answering, in
668 particular by feeding large text corpora into Deep Learning (DL) based Language Models (LMs) (Devlin et
669 al. 2018; Peters et al. 2018; Raffel et al. 2019). These advances have been transferred to protein sequences

670 by learning to predict masked or missing amino acids using large databases of raw protein sequences as input
671 (Alley et al. 2019; Bepler and Berger 2019; Heinzinger et al. 2019; Bepler and Berger 2021; Rives et al.
672 2021; Elnaggar et al.). Such methods leverage the wealth of information in exponentially growing but
673 unlabelled protein sequence databases by solely relying on sequential patterns found in the input. Processing
674 the information learned by such protein LMs (pLMs), e.g., by feeding a protein sequence as input to the
675 network and constructing vectors thereof from the activation in the network's last layers, yields a
676 representation of protein sequences referred to as embeddings (e.g., Fig. 1 in (Elnaggar et al.). This way,
677 features learned by the pLM can be transferred to any (prediction) task requiring numerical protein
678 representations (transfer learning) which has already been showcased for various aspects ranging from protein
679 structure (Rao et al. 2020; Weißenow et al. 2021) over protein function (M. Littmann et al. 2021). Further, it
680 was shown that distance in embedding space correlates with protein function (Maria Littmann et al. 2021)
681 and can be used as an orthogonal signal for clustering proteins into functional families (Littmann et al.).

682 Here, we used the pLM ProtT5-XL-UniRef50 (Elnaggar et al.) (in the following ProtT5) to create fixed-
683 length vector representations for each protein sequence (per-protein embeddings) irrespective of its length.
684 Towards this, we first created individual vector representations for each residue in a protein. In order to derive
685 fixed-length vector representations for single proteins (per-protein embedding) irrespective of a protein's
686 length, we then averaged over all residue embeddings in a protein (Fig. 1 in (Elnaggar et al.). The protein
687 Language Model (pLM) ProtT5 was trained solely on unlabelled protein sequences from BFD (Big Fantastic
688 Database; 2.5 billion sequences including meta-genomic sequences) (Steinegger and Söding 2018; Steinegger
689 et al. 2019) and UniRef50. ProtT5 has been built in analogy to the NLP (Natural Language Processing) T5
690 (Raffel et al. 2019) ultimately learning some of the constraints of protein sequence. As ProtT5 was only
691 trained on unlabelled protein sequences and no supervised training or fine-tuning was performed, there is no
692 risk of information leakage or overfitting to a certain class or label. As a result, every protein was represented
693 as 1024-dimensional per-protein embeddings. Those high-dimensional representations were projected to 3-d
694 using UMAP (n_neighbors=10, min_dist=0.3, random_state=42, n_components=3) and coulored according
695 to their taxonomic group to allow for visual analysis. Embeddings and 3-d plots were created using the
696 bio_embeddings package (Dallago et al. 2021).

## 697 Author contributions

698 BMvR and IK conceived the project. Proteo-transcriptomic and genomic data were analysed by IK, MV and
699 BMvR. Mass spectrometry was conducted by S.D, T.T. and G.L. Machine learning analysis was conducted
700 by M.H. and B.R. New genome and annotation data were provided by E.S., R.G., B.A.H. All authors wrote
701 the manuscript.

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## **Supplementary Material** (See also: https://zenodo.org/record/5734574)

**Supplementary Table 1.** List of described hymenopteran venom proteins and their known functions.
**Supplementary Table 2.** Proteo-transcriptomically identified venom components in *X. violacea*.
**Supplementary Table 3.** Proteo-transcriptomically identified venom components in *H. scabiosae*.
**Supplementary Table 4.** Proteo-transcriptomically identified venom components in *A. mellifera*.
**Supplementary Figure 1.** Phylogenetic tree of anthophilin1 peptides.
**Supplementary Figure 2.** Phylogenetic tree of melittin peptides.
**Supplementary Figure 3.** Phylogenetic tree of phospholipase A2 proteins.
**Supplementary Figure 4.** Phylogenetic tree of hyaluronidase proteins.
**Supplementary Figure 5.** Phylogenetic tree of icarapin proteins.
**Supplementary Figure 6.** Phylogenetic tree of dipeptidyl peptidase 4 proteins.
**Supplementary Figure 7.** Phylogenetic tree of acid phosphatase proteins.
**Supplementary Figure 8.** Phylogenetic tree of venom serine protease proteins.
**Supplementary Figure 9.** Phylogenetic tree of venom allergen proteins.
**Supplementary Figure 10.** Phylogenetic tree of secapin proteins.
**Supplementary File 1.** Alignment of anthophilin1 peptides.
**Supplementary File 2.** Alignment of melittin peptides.
**Supplementary File 3.** Alignment of ant toxins from genomic regions with *Apis* candidate
**Supplementary File 4.** Alignment of "aculeatoxins" with signal peptide
**Supplementary File 5.** Alignment of only mature regions of "aculeatoxins"
**Supplementary File 6.** Machine learning results for "aculeatoxins" with signal peptide
**Supplementary File 7.** Machine learning results for mature region of "aculeatoxins"
**Supplementary File 8.** Alignment of phospholipase A2 proteins.
**Supplementary File 9.** Alignment of hyaluronidase proteins.
**Supplementary File 10.** Alignment of icarapin proteins.
**Supplementary File 11.** Alignment of dipeptidyl peptidase 4 proteins.

**Supplementary File 12.** Alignment of venom acid phosphatase proteins

**Supplementary File 13.** Alignment of venom serine protease proteins.

**Supplementary File 14.** Alignment of venom allergen proteins.

**Supplementary File 15.** Alignment of secapin proteins.

## **Additional material** (ZENODO 10.5281/zenodo.5734574).

**Additional File 1.** Assembly file of *Xylocopa violacea* following ORF prediction by Transdecoder.

**Additional File 2.** Assembly file of *Halictus scabiosae* following ORF prediction by Transdecoder.

**Additional File 3.** Assembly file of *Apis mellifera* following ORF prediction by Transdecoder.

**Additional File 4.** Genome annotation of *Colletes gigas*.

**Additional File 5.** Genome annotation of *Euglossa dilemma*.

**Additional File 6.** Genome annotation of *Melipona beecheii*.

**Additional File 7.** Genome annotation of *Tetragonula carbonaria*.

**Additional File 8.** Gff files of all toxin gene annotations.

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