1 Vexitoxins: a novel class of conotoxin-like venom peptides from predatory
2 gastropods of the genus *Vexillum*

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19 **Abstract**

20 Venoms of predatory marine cone snails (the family Conidae, order Neogastropoda) are
 21 intensely studied because of the broad range of biomedical applications of the neuropeptides that
 22 they contain, conotoxins. Meanwhile anatomy in some other neogastropod lineages strongly
 23 suggests that they have evolved similar venoms independently of cone snails, nevertheless their
 24 venom composition remains unstudied. Here we focus on the most diversified of these lineages,
 25 the genus *Vexillum* (the family Costellariidae). We have generated comprehensive multi-
 26 specimen, multi-tissue RNA-Seq data sets for three *Vexillum* species, and supported our findings
 27 in two species by proteomic profiling. We show that venoms of *Vexillum* are dominated by
 28 highly diversified short cysteine-rich peptides that in many aspects are very similar to
 29 conotoxins. Vexitoxins possess the same precursor organization, display overlapping cysteine
 30 frameworks and share several common post-translational modifications with conotoxins. Some
 31 vexitoxins show detectable sequence similarity to conotoxins, and are predicted to adopt similar
 32 domain conformations, including a pharmacologically relevant inhibitory cysteine-know motif
 33 (ICK). The tubular gL of *Vexillum* is a notably more recent evolutionary novelty than the
 34 conoidean venom gland. Thus, we hypothesize lower divergence between the toxin genes, and
 35 their ‘somatic’ counterparts compared to that in conotoxins, and we find support for this
 36 hypothesis in the molecular evolution of the vexitoxin cluster V027. We use this example to
 37 discuss how future studies on vexitoxins can inform origin and evolution of conotoxins, and how
 38 they may help addressing standing questions in venom evolution.

39 Introduction

40 The order Neogastropoda is a large and successful group of marine gastropod molluscs
 41 comprising over 18,000 described species (MolluscaBase). Most neogastropods are active
 42 predators or blood-suckers (Taylor et al. 1980), and many have developed unique biochemical
 43 innovations to assist hunting and defense (Olivera et al. 2014; Ponte & Modica 2017). The best
 44 known of them are venoms of *Conus* comprising structurally diverse oligopeptides, *conotoxins*,
 45 that cause devastating physiological effects in preys, and may be deadly for humans (Kohn
 46 2018). Due to their ability to selectively block wide array of ion channels in the nervous system,
 47 conotoxins are one of the major highlights in the natural products based pharmacology
 48 (Prashanth et al. 2014; Safavi-Hemami et al. 2019). They are typically short cysteine-rich
 49 peptides, with a high proportion of post-translationally modified residues (Terlau & Olivera
 50 2004). Conotoxin precursors have a uniform structure, comprising a signal sequence, a pro-
 51 region, and a mature peptide domain (Terlau & Olivera 2004; Puillandre et al. 2012). Whereas
 52 signal regions are typically highly conserved, the mature peptide domains evolve under strong
 53 positive selection, and were estimated to be among fastest evolving animal peptides (Chang &
 54 Duda 2012). Whereas cone snail venoms attract broad interdisciplinary interest, the fact remains
 55 barely acknowledged that venoms, likely similar to those in cone snails, are present in some
 56 other neogastropod lineages unrelated to Conoidea.

57 Conotoxins are synthesized in a specialized tubular venom gland, an evolutionary
 58 innovation of the hyperdiverse superfamily Conoidea (Puillandre et al. 2016; Abdelkrim et al.
 59 2018), a homologue of the commonly found in neogastropods mid-gut gland of Leiblein, gL
 60 (Ponder 1973; Kantor 2002). Typically, gL has a spongy structure, and the use of its secretion for
 61 envenomation is unlikely: the duct of gL opens into the mid-oesophagus behind a distinctive
 62 valve of Leiblein (vL), which prevents any particle or fluid transport from mid-oesophagus
 63 anteriorly (Kantor & Fedosov 2009). However, several unrelated neogastropodan lineages beside
 64 Conoidea have evolved a massive tubular compartment in their gland of Leiblein. Its acquisition
 65 was invariantly accompanied by a modification or a complete loss of vL (Ponder 1973; Kantor &
 66 Fedosov 2009; Fedosov et al. 2017), thus effectively setting the stage for venom production and
 67 delivery. Several lines of evidence suggest that each neogastropod lineage possessing such
 68 derived morphology uses venom to subdue and kill the prey (Maes & Ræihle 1975; Olivera et
 69 al. 2014; Fedosov et al. 2019).

70 In the present study, we focus on the most diversified of these lineages, the genus
 71 *Vexillum*. We demonstrate the existence of venom in two *Vexillum* species, based on a
 72 comprehensive transcriptomic analysis of two tissues, supported by proteomic profiling. We

73 show that venoms of *Vexillum* are dominated by highly diversified short cysteine-rich peptides
 74 that we name *vexitoxins* that in many aspects are very similar to conotoxins. Vexitoxins possess
 75 the same precursor organization, display overlapping cysteine frameworks and share several
 76 common post-translational modifications with conotoxins. Some vexitoxins show detectable
 77 sequence similarity to conotoxins, and are predicted to adopt similar domain conformations,
 78 suggesting that they have the same or similar molecular targets. Furthermore, we show that
 79 multiple unrelated vexitoxins contain the inhibitor cystine knot (ICK) motif (Pallaghy et al.
 80 1994), which is present in many pharmacologically relevant animal toxins, including the
 81 conotoxin-based prialt (Robinson & Norton 2014). Therefore, vexitoxins have significant
 82 potential to become a novel source of bioactive peptides for drug development and neuroscience
 83 research.

84 Results

85 General characterization of the transcriptome datasets

86 A total of thirteen transcriptomic datasets were generated for four species of *Vexillum* (Table 1).
 87 Two tissues, salivary gland (sg) and tubular gland of Leiblein (gL) were sequenced with two
 88 replicate specimens for the three species, *Vexillum coccineum* (Vc), *Vexillum vulpecula* (Vv) and
 89 *Vexillum melongena* (Vm). Additionally, a smaller species, *Vexillum crocatum* was sequenced as
 90 a single pooled sample, containing salivary glands and tubular glands of Leiblein of two
 91 specimens (Fig. S1). The generated datasets are similar in terms of the number of reads per
 92 sample and in read quality metrics. The obtained Trinity assemblies were comparable in the
 93 BUSCO completeness (consistently slightly lower in sg compared to the gL of the same
 94 specimen), and were slightly lower in *V. melongena*, compared to *V. coccineum* and *V.*
 95 *vulpecula*. The assembly quality of the latter two species is comparable to that in the
 96 comprehensively sequenced *Conus* datasets (Barghi et al. 2015; Abalde et al. 2018).
 97 Furthermore, the proteomic data was obtained for three individuals of each species, *V.*
 98 *coccineum* and *V. vulpecula* to support and expand the transcriptomic analysis. Therefore, we
 99 mainly focus on the putative venom components identified in these two species, but also discuss
 100 sequences obtained from *V. melongena* and *V. crocatum* where relevant.

101 **Table 1.** Analysed transcriptomic datasets

Species	Spm	Dataset	Total reads	N contigs after clustering	BUSCO Mollusk dataset (%)	BUSCO Metazoa dataset (%)	Mapped to clustered assembly (%)
<i>V. coccineum</i>	#8Vc	#8Vcsg	31,422,354	236,084	27.3	53.8	82.4
<i>V. coccineum</i>	#8Vc	#8VcgL	34,686,405	290,275	34.9	64.7	78.5
<i>V. coccineum</i>	#9Vc	#9Vcsg	32,132,355	249,943	33.9	64.9	83.6
<i>V. coccineum</i>	#9Vc	#9VcgL	29,313,679	302,835	36.1	68.7	79.8
<i>V. vulpecula</i>	#13Vv	#13Vvsg	32,942,762	124,115	20.8	43.3	87.6
<i>V. vulpecula</i>	#13Vv	#13VvgL	37,148,759	212,056	31.7	60.6	81.8
<i>V. vulpecula</i>	#14Vv	#14Vvsg	31,836,954	103,025	17.2	37.5	88.7
<i>V. vulpecula</i>	#14Vv	#14VvgL	26,942,349	291,987	38.3	67.1	77.6
<i>V. melongena</i>	#33Vm	#33Vmsg	34,649,613	74,707	16.1	35.6	82.3
<i>V. melongena</i>	#33Vm	#33Vmgl	32,781,696	149,065	27.1	53.2	82.0
<i>V. melongena</i>	#35Vm	#35Vmsg	38,273,786	87,825	21.0	47.0	85.6
<i>V. melongena</i>	#35Vm	#35Vmgl	36,993,835	124,406	27.1	53.5	84.7
<i>V. crocatum</i>	#44Vr	#44VrsggL	34,535,756	154,716	32.2	60.7	80.8

102

103 The coding DNA sequences (CDSs) predicted from the assembled contigs were filtered to keep
 104 only the CDSs that encode secreted peptides – these start with a C-terminal signal sequence, but
 105 lack a transmembrane domains. A total of 73,945 such CDSs were predicted in four *Vexillum*
 106 species; they were pooled and clustered with two alternative approaches: i) based on the identity
 107 of the signal sequence, with PID 0.65 - (Lu et al. 2020), and ii) based on the orthogroup

inference. In further analysis we focus on the highly expressed clusters of CDS, so we built a reduced data set. If any CDS of a signal sequence based cluster, or of an orthogroup showed a TPM value exceeding 200 in any of the specimens, all members of this cluster or orthogroup, were added to the reduced data set. Thus compiled reduced data set included 3,308 CDSs that were subjected to manual curation to re-classify them to a final set of clusters that would reflect CDS sequence similarity but avoiding cluster oversplitting. We only kept CDS clusters comprising two or more CDSs, so the final data set comprised 235 clusters with 2,187 CDSs. Of these, 850 and 817 CDSs represented putative venom components of *Vexillum coccineum* and *V. vulpecula* respectively.

Figure 1. Phylogeny and morphology of *Vexillum*. A. Mitochondrial phylogeny of the Neogastropoda (after Uribe et al. 2021); the family Costellariidae represented by *Costapex baldwinae*; B. Foregut anatomy of *Vexillum vulpecula*, blue marks the salivary gland (sg), green – tubular gland of Leiblein (gL), grey - proboscis; C. Sampling location; D. Species tree of the four *Vexillum* species analyzed herein based on the ML analysis of concatenated aa sequences of 426 BUSCO loci 126,681 aa sites); E – H. Specimens dissected for transcriptomic analysis; E. *V. coccineum*; F. *V. vulpecula*; G. *V. melongena*; H. *V. crocatum*.

121 *Proteome and peptidome analysis*

122 The main goal of proteomic analysis was to generate support for the venom components
123 predicted based on the transcriptomic data. Because a notable proportion of these putative toxins
124 were predicted to be rather short peptides, and could be passed to mass-spectrometric analysis
125 without a preceding digestion, for each tissue, we analyzed both, the peptidome obtained from
126 the native low molecular weight peptide fraction) and the proteome, generated from the trypsin-
127 digested longer proteins (> 10 kDa).

128 **Table 2.** Results of the proteomic analysis of 12 *Vexillum* samples.

Dataset	Shotgun proteomics			PTMs included		De novo sequencing			Total CDS
	peptides	CD S	Summed peps./CD S	carboxyE peps./CD S	HydroxyP peps./CD S	peptides	CDS	summed peps./CD S	
#3VcgL	510	321				190	168		
#4VcgL	527	329	727/439	104/101	104/146	46	83	254 / 211	479
#6VcgL	402	298				56	71		
#3Vcsg	318	178				23	54		
#4Vcsg	477	291	581/374	51/58	81/101	74	98	180 / 122	390
#6Vcsg	291	225				129	112		
#10Vvg L	423	246				94	74		
#11Vvg L	509	296	619/371	63/70	109/125	55	50	137 / 84	399
#p7Vvg L	350	239				43	48		
#10Vvsg	419	235				158	143		
#11Vvsg	436	244	543/322	25/46	60/93	91	119	262 / 175	349
#p7Vvsg	316	209				124	145		

129
130 The peptidome samples were directly subjected to LS-MS/MS analysis following with *de novo*
131 sequencing by the PEAKS software, while the mass-spectra obtained from the digested samples
132 were searched against the databases derived from the transcriptomic data using conventional
133 proteomic approach (for more details see the Material and Methods section) – Table 2.

134 Among the four analyzed species-tissue series, the gL datasets generated slightly higher
135 number of hits, and no outliers in the hits number were detected in any series. A largest number
136 of 727 unique matches was obtained from the specimens of *V. coccineum* gL, and the lowest
137 (543 matches) from *V. vulpecula* sg. These generated support for 439 and 322 CDS respectively,
138 however a majority of these supported CDS correspond to non-unique matches, because most
139 peptides have generated hits to multiple database entries, which we collectively refer to as
140 “protein group”. When carboxylated glutamic acid and hydroxy-prolyne were set as variable
141 modifications, additional sets of peptides were matched, again with larger numbers of hits in the

gL series, compared to the sg of the respective species. Finally, from 137 to 262 native peptides per tissue-species series were revealed by *de novo* peptide sequencing in the peptidome samples. The largest (479) and the smallest (349) total numbers of supported CDS corresponded to the series of gL of *V. coccineum*, and sg of *V. vulpecula* respectively. We calculated overlaps among samples within each series i) in the detected peptides derived from the trypsin-digested protein fraction (FigS2, top row), and ii) in the subsets of CDSs supported by these peptides (second row). Our results highlight a notable concordance among the analyzed replicates at the CDS level: from 44% to 70% of the supported CDSs, are supported by all three conspecific tissue replicates. Largest contribution to the proteomic support of the query CDSs was generated by the peptides detected with conventional database search from the trypsin-digested protein fraction, however, a sizeable contributions, were also made by the *de novo* protein sequencing, and with modified matching accounting for 2 wide spread in conotoxins PTMs (Fig. S2, bottom row). Subsequently, we aligned all the peptides detected from the matched masses to the matching query CDSs, and summed up the length of predicted mature peptide region of each CDS, supported by the detected peptides. This value was divided by the total length of the predicted mature region, and the resulting ratio used as a measure of support; we report it for three best supported CDSs of each putative toxins cluster inferred from the transcriptomic data. In 31 and 25 CDSs of *Vexillum coccineum* and *V. vulpecula* respectively, obtained proteomic data was also essential to correct predicted boundaries of the mature peptide region.

Venom composition in *Vexillum*

Confident BLAST or HMMER hits were obtained for 309 and 294 CDSs of *V. coccineum* and *V. vulpecula* respectively, which constitute 36.4% and 36.0% of the putative venom components in these two species respectively (Fig. 2A). The transcripts with reference-based annotations belonged to 47 Pfam gene families. Proteins bearing shaker toxin (ShKT) domains and metalloproteases, mainly of astacin type were the most diversified of annotated clusters in both, the sg and the gL of both species (Figs 2B, C). Both, ShKT domain bearing proteins, and astacins showed high expression in both tissue types, with notably higher total expression levels of ShKT domain bearing proteins in sg (Fig. 2D). Other highly expressed classes of venom peptides included Prohormone-4, peptidases –S, –M, and –C, and CRISP, Lectin-C, hyaluronidases, chitinase (CBM_14), Abhydrolase, serine type protease inhibitors (ITIH3, Pacifastin, Serpin), von Willebrand domain bearing proteins.

The small number of available replicates precluded statistically sound differential expression analysis; however, the contrasting expression levels in some venom components clusters can be noted (Fig. S3). For example, abhydrolase, CRISP, DNase1, Exo-endo-phos,

176 hyaluronidase, Pacifastin and ShKT bearing proteins show higher expression in sg (Figs 2D, S3).
 177 On the contrary, cerebrin, DUF1091, Ependymin, Laminin G1, Lectin C, PLAT-type
 178 metalloprotease, serpin and notably prohormone-4-like transcripts display higher expression in
 179 the gL.

180

Figure 2. Major annotated clusters of transcripts in the sg and gL of *Vexillum* species. A. Proportions of annotated and unannotated transcripts in *Vexillum coccineum* (left) and *V. vulpecula* (right); B. Ten most diversified classes of annotated transcripts in salivary gland (sg), pink - *Vexillum coccineum*, yellow - *V. vulpecula*; C. Ten most diversified classes of annotated transcripts in gland of Leiblein; D. Heatmap of log10 transformed summed TPM expression levels of 30 most highly expressed annotated transcript classes per data set. On the right heatmap of the cluster support in proteomic data, where three cells in a horizontal row correspond to three CDS of a cluster best represented in our proteomic data. Color-coding corresponds to the proportion of the mature peptide length, represented in the proteomic data.

181

182 While prohormone-4, lectins, CRISP, and hyaluronidases (as conohyal) have previously been
 183 identified in *Conus* venoms (Robinson et al. 2017; Fassio et al. 2019; Lebbe & Tytgat 2016),
 184 other *Vexillum* venom components are not typically reported from cone snails. Nevertheless, at
 185 least some of them: astacins, ShK-domain bearing proteins, peptidases, ab-hydrolases, serine-
 186 protease inhibitors are present in venom gland transcriptomes of the early-diverging cone snail
 187 lineages *Profundiconus* (Fassio et al. 2019), *Conasprella* and *Pygmaeonus* (Fedosov et al.

2021). Some of these transcripts typically show lower expression in cone snails, and were suggested to play an accessory role in envenomation, by facilitating spread of venom, or impairing the prey's hemostasis (Fassio et al. 2019). The presence of these putative venom components in both the sg and gL of *Vexillum* as evidenced by both transcriptomic and proteomic data, suggests that secretions of both these glands play a role in envenomation. However, functional aspects of *Vexillum* venom components are still to be determined, and the priority here will be given to the putative toxins that we cover in further detail below.

195

196 *Proteins bearing ShKT domains are diversified and highly expressed in Vexillum*

197 We identified a total of 98 complete transcripts of ShKT bearing proteins that can be classified to
198 three gene superfamilies based on the identity of their signal sequence (Fig 3A). Because most
199 predicted transcripts bear multiple ShK domains, we denote these clusters here as multiShKV1 –
200 VexShKV3 (Gerdol et al. 2019). The members of these three gene superfamilies show major
201 differences in both the numbers of ShKT-like domains that they comprise and the regions
202 interleaving these domains. The only complete precursor of the small multiShKV1 gene
203 superfamily, Vc00003648 is predicted to bear five ShKT-like domains (Fig. 3B). The three N-
204 terminal domains show only limited identity to the canonical ShKT domain (HMMER evalue <
205 E*10⁻², and lack one or two cysteines). The transcripts of the large multiShKV2 gene
206 superfamily encode up to five (e.g. Vv0001310, Fig. 3C), but typically three ShKT-like domains
207 (e.g. Vc0000421, Fig. 3D). These transcripts feature a long low-complexity region with high
208 proportion of charged (both positively and negatively) residues between the signal sequence and
209 first ShKT-like domain. Finally, the majority of the multiShKV3 gene superfamily transcripts
210 comprise only two ShKT-like domains (Figs 3E, F), and also contain a low-complexity region.
211 This region spans up to 140 residues, and is composed of repeated short motif, starting with two
212 negatively charged residues (typically DE), followed by 2-7 neutral residues. The log₁₀
213 transformed expression of the ShKT bearing proteins (Fig. 3A) shows transcripts' expression in
214 sg (blue) and gL (green), with the circular line marking a TPM 1000 expression (grey used for *V.*
215 *crocatum* where the glands were pooled). It can be noted that multiShKV2 and multiShKV3
216 show contrasting expression patterns: the former is represented by about equal number of
217 transcripts in sg and gL, but the highest expression transcripts are those in gL. Conversely, the
218 multiShKV3 is dominating sg in both, the number of transcripts, and in their expression levels.

219

Figure 3. MultiShK proteins of *Vexillum*. A – E. Domain arrangement in five representative transcripts. A. Vc0003648 (multiShK1); B. Vv0001310 (multiShKV2); C. Vc0000412 (multiShKV2); D. Vc0000028 (multiShKV3); E. Vc0000358 (multiShKV3); F. Phylogenetic tree of the 98 identified complete multiShK protein precursors. The annotation corresponds to the log10 transformed TPM expression levels, shown in blue for sg, in green for gL, in grey – for polled tissues of *V. crocatum*. Outer circular line marks TPM expression level of 1000. G-L. Predicted 3D structures and inferred disulphide connectivity of the six structure types of *Vexillum* ShKT domains supported by proteomic data. G. Type I, Vc0000028, domain 1; H. Type II, Vc0000028, domain 2; I. Type III Vc0000358 domain 1; J. Type IV Vc0005635 domain 2; K. Type V Vv0001739 domain 2; L. Type VI Vc0005911 domain 2.

220 Previously identified ShK toxins of sea anemones are short neuropeptides, comprising six
 221 cysteine residues (Castañeda et al. 1995). They are potent potassium channel blockers, with high
 222 affinity to channels comprising a Kv1 subunit, especially of the Kv1.3 subtype (Pennington et al.
 223 1995; Kalman et al. 1998). This makes them a valuable source of drug leads modulating immune
 224 functions: the Kv1.3 channels are crucial for terminally differentiated effector memory (TEM) T
 225 cells functioning, which are responsible for a wide range of autoimmune conditions. Many ShK
 226 toxins therefore have been chemically synthesized, and proved efficient in animal models of
 227 human autoimmune diseases (Chi et al. 2012; Tarcha et al. 2017). Of the total of 33 and 29
 228 unique ShKT-like domains predicted in transcripts of *Vexillum coccineum* and *V. vulpecula*
 229 respectively, 17 and 12 respectively were supported by the proteomic data – all these are the
 230 domains encoded by the transcripts of multiShKV2 and multiShKV3 gene superfamilies. A total
 231 of 62 unique monoisotopic masses were detected in the proteomic datasets of *V. coccineum* that
 232 match the ShKT-like domain sequences, and a total of 31 unique masses support the *V. vulpecula*

233 ShKT-like domains. We obtained high confidence 3D structure models (LTTD score typically
234 above 90) for all the identified ShKT-like domains supported by the proteomic data (Figure S4).
235 They demonstrated a high diversity of conformations that we classify into six general structural
236 types, referred to as types I-VI (shown in the figures 3 G-L in the order of decreasing expression
237 levels of the respective transcripts). The most common structure types I and II (Figs 3G, H) are
238 encoded by both the multiShKV2 and multiShKV3 transcripts, and geometrically the closest
239 match of both is the shaker toxin k of *Stichodactyla helianthus* (Figure S4). The structure types
240 III, IV and V (Figs 3I-K), although share general features of ShKT domains, geometrically show
241 higher resemblance to the pseudocins – the CRISP class toxins of Elapidae snakes targeting
242 cyclic nucleotide-gated ion channels (Suzuki et al. 2008). Finally, one ShKT-like domain
243 detected in *V. vulpecula* transcript V0001739 (Fig 3L) shows high structure resemblance to the
244 natrin, a potent blocker of calcium-activated potassium (BK(Ca)) channels (Wang et al. 2005).

245 Although we do not have any direct evidence of the physiological activity of *Vexillum*
246 ShK-like peptides, our data points at ion channels as their tentative targets. Indeed, the presence
247 and the remarkable diversity of ShKT bearing proteins is predicted by the transcriptomic data of
248 multiple species and specimens, and is further corroborated by the mass-spectrometric analysis.
249 The very high expression of these transcripts in the secretory foregut glands suggests their
250 significant role in the context of functionality of salivary glands and of the gland of Leiblein –
251 i.e. presumably in envenomation. Finally, the detected sequence similarity of the *Vexillum*
252 ShKT-like peptides with the sea anemone ShK toxins, and with the ion channel blockers of
253 snake venoms potentially suggest that the ShKT-like peptides of *Vexillum* share same range of
254 targets.

255 *Unannotated clusters of transcripts*

256 The majority of the predicted secreted CDSs did not display any sequence similarity to the
257 entries in the reference databases. Here we consider them together with the total of 32 CDSs that
258 showed structure similarity with conotoxins (of these ten in *V. coccineum* and seven in *V.*
259 *vulpecula*). The reason for it is that a large set of unannotated CDSs appears to share
260 characteristic features of conotoxins, therefore the entire diversity of putative conotoxin-like
261 transcripts is analyzed in the context of this similarity. These features are: i) the canonical
262 precursor structure with a conserved signal sequence, and a rather short, variable mature domain
263 represented by a single copy; ii) high number of cysteine residues in the mature domain that
264 form distinctive cys-frameworks; iii) high number of post-translationally modified residues in
265 the mature region.

Figure 4. Expression and structural features of the unannotated *Vexillum* transcript clusters. A. heatmap of log 10 transformed expression of 118 unannotated clusters of transcripts in sg and gL transcriptomes of *Vexillum coccineum* and *V. vulpecula*. Column pr – support of clusters in proteomic data (markup like in Figure 2). Column c: grey marks presence of a conserved cys-framework across the sequences of a cluster, or of several compatible frameworks. Columns p1 – p3 – prediction of three PTMs most commonly found in conotoxins: p1 – N-terminal pyroglutamic acid, p2 – carboxy-glutamate, p3 – C-terminal amidation. B. Most common Cys-frameworks in unannotated clusters of putative *Vexillum* toxins. C – E. Mature peptide alignment in three clusters of vexitoxins with closest conotoxin matches. C. Cluster V041. D. V117. E. V027. F. Superposition of the vexitoxin Vc0001332 VS *Conus tulipa* conotoxin p-conotoxin TIA. G. Superposition of the vexitoxin Vc0004145 VS *Conus geographus* conotoxin GS. H. 3D structure of the vexitoxin Vv0000573. I. Heatmap of log 10-transformed expression level of seven key PTM enzymes in analyzed transcriptomes of *Vexillum coccineum* and *V. vulpecula*.

267 The entire diversity of 1,580 unannotated secreted CDSs was classified into 146 clusters based
 268 on both, the identity of their signal sequences and the orthogroup inference; each cluster was
 269 assigned a digital code based on its summed expression (Table S1), supplemented by letters to
 270 reflect i) length of its constituent CDSs, and ii) degree of their sequence conservation. In total,
 271 117 of these clusters demonstrated high expression in at least one of the profiled specimens
 272 ($\text{TPM} \geq 1000$), or moderately high expression ($\text{TPM} \geq 100$) across several specimens. In Figure
 273 4A, the horizontal rows of cells that summarize cluster expression, are arranged based on the
 274 length of CDSs included in a cluster, and degree of sequence variation within a cluster (see
 275 colored ranges on the left). The clusters V001, V002, and V004 showed highest sequence
 276 diversity and extremely high expression in all analyzed datasets (Table S1). All three clusters
 277 appeared very heterogeneous. Despite the fact that sequences in each of them share a conserved
 278 signal sequence and recognizable sequence motifs in pre- and mature regions, each cluster
 279 included several distinctive major orthogroups (Figs S5-S7). In general, each of these three
 280 clusters showed notably higher expression levels in sg compared to gL (Fig 4A). Otherwise, it
 281 can be noted from the figure 4A that the clusters of medium-sized CDSs (entire precursor longer
 282 than 40 aa, but shorter than 200 aa), with over 10% variable aa sites (blue bar on the left) are
 283 much broader represented in gL than in sg.

284 In the column 'c' of Figure 4A, we highlighted in grey those clusters, where mature
 285 regions of complete CDSs comprise at least two cysteine residues, and share the same or display
 286 compatible Cys-frameworks across each cluster (except V001, V002, and V004, where some
 287 variation was permitted). Fifty-five clusters can be considered as sharing structural features of
 288 conotoxins: they comprise cys-rich precursors whose length matches the length range of
 289 conotoxins. Of a total 942 complete transcripts in these clusters, 445 (or almost half) encode
 290 mature toxins with canonical Cys frameworks known from conotoxins. Of the 14 most common
 291 frameworks that are shared by no less than 10 predicted CDSs, nine are canonical frameworks
 292 known in conotoxins (Fig. 4B). For example, the framework IX found in 119 *Vexillum* CDSs is
 293 present in most P-superfamily conotoxins (Fedosov et al. 2012; Robinson et al. 2014), and the
 294 framework VI/VII, known also as the inhibitor cysteine knot (Robinson & Norton 2014;
 295 Lavergne et al. 2015), is most common in the O-, H- and N- conotoxin superfamilies. Two
 296 further frameworks marked with an asterisk are rather exotic for conotoxins (Lavergne et al.
 297 2015). The VI/VII framework shared by 71 identified putative toxins of *Vexillum* is the third
 298 most common in our data set. The O1-superfamily conotoxins with the framework IV/IIV are
 299 potent blockers of voltage gated ion channels targeting Na^+ channels (pharmacological families
 300 δ^- , and μ^-), K^+ channels (κ^-), and Ca^{2+} channels (ω^-), and therefore are of great interest for drug
 301 development (Robinson & Norton 2014; Safavi-Hemami et al. 2019). In particular, the first

conotoxin approved by FDA for clinical use the ω -conotoxin MVIIA (Prialt) possesses this cys-framework. The remarkable sequence diversity of framework IV/IIIV toxins in *Vexillum* may suggest a similar scope of their molecular targets, and if proved true, *Vexillum* toxins may become a rich source of neuropeptides of high relevance for biomedical research and drug development.

The mature toxin alignments of three clusters that have displayed detectable similarity to conotoxins are showed in Figures 4C-E, their disulphide connectivity was inferred from the reconstructed high confidence 3D models (Figs 4E-H, respectively). The CDS Vc0001332 (cluster V041) has a rather uncommon cys-framework XI with four disulfide bounds. While its predicted sequence is closest to that of *Conus pulicarius* I3-superfamily conotoxin Pu11.2 (Fig 4C), the core of the predicted structure shows highest similarity to the much shorter ρ -conotoxin TIA (A-superfamily) of the fish-hunting species *Conus tulipa* (Fig 4F). The putative *Vexillum* toxins Vc0004145 and Vv0000573 both contain a ICK motif with its signature connectivity 1-4, 2-5, 3-6 (Figs 4G, H), and show closest sequence similarity to the S6.7 of *Conus striatus*, and to Om6.1 of *Conus omaria* respectively (both O1-superfamily). The modeled 3D structure of the Vc0004145 showed a close match to that of the synthetic μ -conotoxin GS (Hu et al. 2012) of *Conus geographus* (Fig 4G). Finally, some longer *Vexillum* toxins, such as the Vv0000706 (cluster V064), and Vc0004790 (V136) contain 12 cysteins which are predicted to fold into two ICK-like structures. The structure search on the obtained PBD files detected their highest structure similarity to the cyriotoxin-1a of the spider *Cyriopagopus schioedtei* (Fig. S7).

To estimate, whether the predicted *Vexillum* toxins bear same PTMs as do conotoxins, we summarized the PTM predictions obtained from ConoPrec (Fig 4A, columns p1 – p3), and corroborated these by the expression data of the corresponding PTM enzymes in the sg and gL transcriptomes (Fig. 4I). Our results suggest that these PTMs are likely to be quite common in *Vexillum* toxins. Among the predicted PTMs, the gamma-carboxylated glutamate was most commonly predicted (395 putative toxins from 56 clusters), however, the reliability of this PTM prediction from the primary protein sequence is questionable (Shah & Khan 2020). When glutamate carboxylation was set as a variable modification to expand the search of MS data, we recovered 25 to 104 additional unique monoisotopic masses per tissue-species series, with larger number of additional hits in gL compared to conspecific sg samples (Table 2). This suggests that if not a most common PTM, gamma-carboxylated glutamate at least occurs with a detectable frequency. The N-terminal amidation was predicted as the second most common PTM (236 putative toxins from 32 clusters). Furthermore, we detected presence of the seven essential PTM enzymes in the analyzed transcriptomes (Fig 4I). Protein disulfide isomerase, prolyl 4-

hydroxylase (P4H), and peptidyl-prolyl cis-trans isomerase (PPI) showed highest expression levels. There is a clear pattern with higher expression of all these enzymes in the gL compared to sg. Glutamyl-peptide cyclotransferase (GPC), Vitamin K-dependent carboxylase (VKD), and peptidyl-glycine amidating monooxygenase (PAM), were detected in all gL transcriptomes, but the former two were lacking in two sg data sets. Nevertheless, there is no tissue-specific pattern in the expression of the latter three enzymes. The presence of these essential PTM enzymes in most analyzed transcriptomes supports the hypothesis that peptide products of sg, and particularly, gL feature same post-translational modifications as conotoxins.

Cross-tissue recruitment exemplified by the V027 cluster evolution

A close inspection of the cluster V027 sequences revealed that they represent two orthogroups with considerable differences in sequence length and tissue expression specificity. The first orthogroup sequences are about 230 aa long and are expressed predominantly in salivary glands, with the expression levels varying among species. The second orthogroup sequences are 92-93 aa long, and are only detected in gL of *Vexillum coccineum* and *V. vulpecula* with a very low expression in the former, and conversely, a fairly high expression in the latter (TPM~5000). Both orthogroups share a high identity N-terminal signal sequence, and a short C-terminal 35 aa long fragment, with a ICK motif. The observed length difference between them is due to the presence in the first orthogroup sequences of a conserved 111 aa long region annotated as a 'frizzled' domain by HMMER, and showing highest sequence similarity to the cys-rich domain of the FZD4 protein (Zhang et al. 2011). The first orthogroups sequences are predicted to cleave into two fragments, one corresponding to the N-terminal signal sequence, another one to a long C-terminal mature peptide combining the Fz-domain with its flanking regions, and the ICK-bearing domain. On the contrary, the second orthogroup sequences are predicted to be cleaved in a manner similar to conotoxin precursors: into three fragments, corresponding to i) a signal sequence, ii) a short pro-region, and iii) a short mature peptide, which exactly corresponds to the N-terminal ICK-bearing domain. This mature peptide sequence shows a detectable similarity to the omega-conotoxin Om6.1 of *Conus omaria* (Fig. 4E), and molecular modeling predicted it to adopt a conformation characteristic for omega toxins family (Fig. 4H). Three monoisotopic masses, uniquely matching the Frizzled domain aa sequence are detected in proteomic data on *V. coccineum*. Consistent with transcriptomic data, these peptides were present in all six analyzed samples (i.e. in both, the sg and gL), but were entirely lacking in *V. vulpecula*. On the contrary, peptides matching the V027 ICK motif were only detected in the gL samples of *Vexillum vulpecula* (#11VvgL, #p7VvgL), and were represented by two nearly identical peptides (16 aa and 17 aa long) that match the N-terminal half of the ICK of the transcript Vv0000805.

Figure 5. Precursor structure and evolution of the cluster V027 sequences in *Vexillum*. A. Precursor structures in V027. Top - long orthogroup, Vr0003450; Middle – long orthogroup, Vc0001640; Bottom – short orthogroup Vv0000573. B. Codons under negative selection (red), pervasive positive selection, identified by FEL, FUBAR, or SLAC, or episodic diversifying selection (identified by MEME) (green).

371

372 The reconstructed phylogeny of the V027 cluster sequences (Fig S8) and the orthogroups
373 distribution across species suggest that the first (longer) orthogroup transcript structure is
374 ancestral, and the one of the second orthogroup is derived. The selection analysis identified 17
375 sites of the precursor under the negative selection, and these sites are predominantly located in
376 the Fz domain. Conversely, of eight sites identified across the alignment, subject to either
377 pervasive positive selection (FEL, SNAP, FUBAR), or evolving under diversifying selection
378 (MEME), five are within the ICK-bearing domain.

379 This pattern is consistent with the second orthogroup descending from the first one resulting
380 from a gene duplication event that has occurred before the split of *V. coccineum* and *V.*
381 *vulpecula*. Following the gene duplication, the second orthogroup sequences lost the Fz-domain,
382 and acquired a cleavage site at the N-terminal boundary of the cys-rich region. Subsequently, the
383 shortened mature peptide region was rapidly evolving under positive selection and gained high
384 tissue-specific expression in gL of *Vexillum vulpecula*. The very high expression of the
385 transcripts Vv0000573 and Vv0000805 in *V. vulpecula* gL, presence of the matching translation
386 products in the proteome, and their 3D structure determined by the ICK, all point at the

387 relevance of this cluster to envenomation. This example illustrates how the cross-tissue
388 recruitment of a gene copy followed by its accelerated sequence evolution gives rise to a
389 pharmacologically relevant venom component following predators' speciation.

390 Discussion

391 *Vexillum* toxins a novel source of bioactive neuropeptides

392 The molecular targets of conotoxins – a wide array of ion channels, and receptors in nervous
 393 system and at neuro-muscular junction have made them promising source of analgesics and a
 394 potentially preferable treatment for long-term pain management (Safavi-Hemami et al. 2019).
 395 The relevance of conotoxins as pharmacological agents can be explained by the fact that venoms
 396 in some cone snail species were evolved specifically to subdue vertebrate preys (Olivera et al.
 397 2014, 2015). In this perspective, the fish-hunting species of *Conus* (or more broadly, those
 398 venomous lineages that are specialized to hunt vertebrate preys), are the first priority for drug-
 399 discovery. While this logic formulates a ‘pragmatic’ approach to prioritizing targets of resource-
 400 consuming drug development process, it would lead to *a priori* elimination of many potentially
 401 valuable candidate molecules. For example, the α -conotoxin Rg1a acting as an inhibitor of the
 402 $\alpha 9\alpha 10$ nicotinic acetyl-choline receptors (nAChR), proved to be a potent analgesic (Bjørn-
 403 Yoshimoto et al. 2020; L et al. 2014), despite being produced by a worm-hunter species *Conus*
 404 *regius*. Similarly, sea anemones do not feed on vertebrate preys, nevertheless, ShK toxins have
 405 high affinity to the vertebrate subtypes of potassium channels (Pennington et al. 1995). These
 406 examples may be explained by either broad taxonomic distribution of relevant molecular targets,
 407 or by the existence of defensive components of venoms, which evolve to efficiently deter
 408 vertebrate predators, rather than to subdue a prey. The defensive venoms targeted to vertebrates
 409 may have a much broader distribution across animal lineages, compared to the predatory toxins
 410 targeted to vertebrates. Furthermore, the ancestral defensive venom compounds are believed
 411 have become the substrate for the evolution of a novel predatory toxin set enabling piscivory in
 412 *Conus* (Imperial et al. 2007). In this context, a broader sampling of venomous animal taxa is
 413 crucial to systematically explore their molecular adaptations to hunting and, as well, to defense,
 414 and to efficiently reveal novel bioactive compounds of potential interest for pharmacology.

415 In the present study, we make a first step towards documenting venom composition of a
 416 highly diversified, yet previously unexplored lineage of venomous gastropods, the genus
 417 *Vexillum*. Due to the small size of its glands compared to a venom gland of cone snails,
 418 collection of sufficient material for bioassays of *Vexillum* venoms is a challenging task. To
 419 overcome this challenge, we used RNA-Seq and shotgun proteomics approaches that both
 420 require little material to generate a high-quality multi-tissue, multi-specimen, and multi-species
 421 data set to enable a rigorous analysis of *Vexillum* venom composition. We uncover highly
 422 diversified short secreted peptides referable to CRISP neuropeptides class in both, the salivary
 423 gland, and specialized tubular gland of Leiblein of *Vexillum*. One distinct group of these

424 neuropeptides are the shaker-like toxins. These are synthesized as multiShK proteins that
425 constitute two highly diversified unrelated multigene families with contrasting expression
426 patterns in sg and gL of *Vexillum*. The ShKT domains encoded by these proteins share crucial
427 structural features of the sea anemones ShK toxins, and therefore are likely to share similar or
428 related molecular targets. Because ShK toxins of sea anemones have proved efficient in
429 treatment of some autoimmune conditions (Chi et al. 2012), due to their high affinity to Kv1.3
430 potassium channels, *Vexillum* counterparts of the ShKTs represent an interesting group of short
431 peptides for further pharmacological characterization.

432 We analyze the vast diversity of short Cys-rich secreted peptides of *Vexillum*, for which no
433 reference-based annotation could be retrieved, in the context of their similarity with conotoxins.
434 In total 55 transcript clusters (47 supported by proteomic data), show structural features
435 characteristic of animal toxins (in particular, of conotoxins): short mature domain, largely
436 conserved Cys-framework, and the presence of some signature PTMs. Of them 141 and 127
437 complete transcripts were identified in *Vexillum coccineum* and *V. vulpecula* respectively that
438 share canonical Cys-frameworks of known classes of conotoxins. These numbers fall well in the
439 range of the per-species conotoxin diversity assessed from the venom gland transcriptomes of
440 *Conus* (Fassio et al. 2019). Although we do not have functional data to support the claim that
441 these predicted transcripts indeed encode potent toxins, we present strong evidence that i) their
442 translation products do exist in the protein fraction of analysed secretory glands, and ii) structural
443 features strongly suggest that at least a sizeable fraction of them are toxins. It is thus logical to
444 propose that they target same physiological circuits of preys and predators as do the conotoxins.
445 Therefore, by this study we establish a solid background for the subsequent functional
446 characterization of identified *Vexillum* toxins.

447 *Comparative framework for venom evolution inference in Neogastropoda*

448 Venoms have evolved over hundred times in independent metazoan lineages (Schendel et
449 al. 2019), offering a unique opportunity for studying genetic underpinnings of repeated key traits
450 apparition (Casewell et al. 2013; Zancolli & Casewell 2020). Being a key adaptation for
451 predation and defense, venoms to a great extent affect species fitness and biology (Dutertre et al.
452 2014; Casewell et al. 2017). Setting up venom production requires novel specialized tissues and
453 glands, in which a set of genes originally not related to the venomous function is recruited and
454 modified to encode potent toxins. Most animal toxins represent rather few broad classes of
455 proteins, such as cysteine rich secretory peptides (CRISPs), hyaluronidases, kunitz-
456 phospholipase and serine-type proteases (Zancolli & Casewell 2020), but being broadly
457 distributed across unrelated venomous animal taxa, they have been recruited from very different

458 genomic backgrounds (Barua & Mikheyev 2021). This general trend to convergent evolution
459 provides a unique opportunity to disentangle the interplay of lineage-specific and conserved
460 mechanisms that govern recruitment and evolution of venom peptides. To enable such inference
461 globally, a scalable comparative framework should be generated to cover entire phylogenetic
462 diversity of venomous animals. Notwithstanding, taxonomically restricted fragments of such
463 framework may yield deep insights into genomic underpinnings of evolution and regulation of
464 venomous function. Currently, most efforts to this end focus on the well characterized taxa of
465 venomous animals, mainly on snakes (e.g. Barua & Mikheyev 2019, 2021), and extending such
466 studies to new system(s) will greatly magnify the power of comparative analysis. Essentially,
467 such system can be seeded by a pair of distantly related taxa that have independently acquired
468 venom function, and cone snails and *Vexillum* representing unrelated evolutionary successful
469 radiations of venomous neogastropods are thus a perfect system.

470 Evolutionary histories and distributions of *Conus* and *Vexillum* display multiple parallels.
471 Similar to *Conus*, *Vexillum* is species rich (encompassing about 390 species), and forms a crown
472 group of its respective family, the Costellariidae (Kohn 1990; Fedosov et al. 2017). Similar to
473 *Conus*, *Vexillum* underwent a major diversification in Miocene, and its present day diversity is
474 mainly associated with tropical shallow waters of Indo-Pacific. Therefore, the adaptive radiations
475 of *Conus* and *Vexillum* were likely shaped by the same set of factors, and acquisition of venom
476 likely have played a major role in the success of both these taxa. Within this system, repeated
477 recruitments of a novel specialized secretory tissue of gL allows comparative analysis of the
478 genome evolution processes underpinning emergence of venom gene superfamilies, and
479 establishment of their regulatory pathways. Because tubular gL has the same developmental
480 origin in *Vexillum* and *Conus* (as stripped off dorsal oesophagus wall), the gene expression
481 patterns in the ancestral tissues were presumably closely comparable among them. Conversely,
482 sg is homologous **and** morphologically conserved across Neogastropoda, and also produces
483 some classes of bioactive compounds in both cone snails and *Vexillum* (Ponte & Modica 2017;
484 Biggs et al. 2008, the present study). This two-tissue system enables a comparative analysis of
485 modes and tempos of molecular evolution, as well, as investigation of cross-tissue gene
486 superfamilies recruitment between sg and gL. In the present study, we demonstrate an example
487 of the sg-gL cross-tissue recruitment in the *Vexillum* V027 cluster.

488 After an initial gene duplication, the gene structure of the new paralog was modified to
489 produce a short ICK-bearing toxin. Subsequently, after the divergence of *Vexillum coccineum*
490 and *V. vulpecula*, the toxin sequence evolved under accelerated positive selection and gained
491 high expression in the gL of the latter species. Interestingly, the mounting expression of this

492 toxin in gL of *V. vulpecula* was accompanied by the reduction of the ancestral (long) paralog
 493 expression in the sg, suggesting that the functionality of their gene products may to some extent
 494 overlap. What we find remarkable in this example is that we were still able to capture the low
 495 expression counterpart of the ancestral (long) orthogroup in the sg of *V. vulpecula*.
 496 Furthermore, we detected a low-expression ‘prototype’ of the ICK-bearing toxin gene (the
 497 short orthogroup) in the gL of *V. coccineum*, where it is expressed alongside the ancestral
 498 orthogroup, but with an order of magnitude lower expression level. It is likely that these low-
 499 expression counterparts are not functional in the context of the biology of the respective
 500 species, and their observed expression is residual, and would have completely vanish if the
 501 divergence between *V. coccineum* and *V. vulpecula* was less recent.

502 The observed distribution of orthogroups across tissues and species of *Vexillum*, as well,
 503 as the distribution of ShKT-bearing transcripts, imply that there remains some functional
 504 overlap between the sg and gL in *Vexillum*, in relation to envenomation. If true, such overlap
 505 may generate a ‘highway’ for cross-tissue recruitment of venom components in the
 506 evolutionary young gL (Fedosov & Kantor 2010; Fedosov et al. 2017) by means of
 507 subfunctionalization (Hargreaves et al. 2014). Therefore, a sizeable fraction of venom
 508 components in *Vexillum* is likely to result from recent recruitment events, and so, despite the
 509 inherent quick divergence from the ancestral state, the structural or sequence similarity of these
 510 venom genes with their non-venomous paralogs may still be traceable. If this is true, *Vexillum*
 511 venoms provide an ideal system to study origin and early evolution of venomous function in
 512 general. Furthermore, outcomes of this analysis have a great potential to inform the evolution
 513 of conotoxins. Genomic source of conotoxin genes origin remains unknown, mainly because
 514 these genes evolve too fast, and the venomous function has originated in the ancestors of cone
 515 snails too long ago (Abdelkrim et al. 2018) for detection of the toxin genes ancestry to be
 516 possible. However, because *Vexillum* and *Conus* share a common ancestor within the
 517 Neogastropoda, their genomic background is largely the same. Therefore, venom evolution
 518 inference in *Vexillum* will give a shortcut to identifying the set of ancestral neogastropod genes
 519 amenable for venom function, and this knowledge, in turn, will generate sensible hypotheses on
 520 the evolutionary origin of conotoxins.

521

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528

529 **Data availability**

530 The transcriptomic sequencing data are deposited in the NCBI SRA database, under the
531 Bioproject PRJNA797643. Sequences of the predicted *Vexillum* toxins are provided in the
532 supplementary data files. The essential Python scripts used for the data analysis are available at
533 <https://github.com/SashaFedosov/Vexillum/>.

534

535 **Material and methods**

536 *Specimen collection and tissue sampling*

537 Specimens of four *Vexillum* species were collected by SCUBA diving in Nha-Trang Bay, Central
 538 Vietnam in May 2021. Five specimens of *V. coccineum* measuring 54 - 58.5 mm, five specimens of *V.*
 539 *vulpecula* (60 – 67.5 mm), and two specimens of *V. melongena* (51.5 and 52.8 mm) were collected at
 540 depths 5-8 meters in Dam Bay (Tre Island) on silted sand. Two specimens of *V. crocatum* (22.3 and 28.4
 541 mm), were collected in a crevice of a vertical reef wall at depth 12 meters off Noi Island. All specimens
 542 were delivered in the onshore laboratory and kept in tanks with aeration overnight; dissections were
 543 performed on the following day. Two specimens of each species were dissected for transcriptomic
 544 analysis, and three additional specimens were dissected for each, *V. coccineum* and *V. vulpecula* for
 545 proteomic analysis. Prior to the dissection, each specimen was photographed, then a vise was used to
 546 destroy the shell, and the body was promptly dissected to excise the salivary gland (sg) and the tubular
 547 gland of Leiblein (gL). These were preserved individually, for each specimen except *Vexillum crocatum* –
 548 for the latter species two sg and two gL were pooled in a single sample (44VrsggL). Tissues for
 549 transcriptomic analysis were preserved in RNAlater (ThermoFisher), kept 24 hours at room temperature,
 550 and then stored at -20°C until dissection. Samples for proteomic analysis were immediately frozen in
 551 liquid nitrogen, and kept at -70°C until further processing. A fragment of foot was clipped from each
 552 dissected specimen and preserved in 95% ethanol to confirm species identity by means of DNA-
 553 barcoding.

554 *RNA Isolation, and sequencing*

555 RNA was extracted from sg and gL tissues of *Vexillum* using the standard Trizol method. Bioanalyzer
 556 traces were used to assess total RNA quality and determine suitability for sequencing. The cDNA
 557 libraries for Illumina pair-end sequencing were then prepared following the automated polyA RNAseq
 558 library prep protocol. All libraries were sequenced on the Illumina HiSeq 4000 platform, at the sequencing
 559 facility 'Genoanalitica' (*V. coccineum* and *V. vulpecula*), or at the genomics core facility of Skolkovo
 560 Institute of Science and Technology (*V. melongena* and *V. crocatum*).

561 *Transcriptome assembly and reference based annotation*

562 The raw reads were quality checked using FastQC, and then filtered to remove putative contamination by
 563 running FastQ-Screen v0.14.1 (Wingett & Andrews 2018), with Bowtie2 (Langmead & Salzberg 2012)
 564 mapper. The reads were mapped to 26 genomes, including those of Human, mouse, yeast, *Drosophila*,
 565 *Arabidopsis*, *E. coli* and *Cutibacterium acnes*, as well, as to the genomes of the other organisms that were
 566 library-prepped, or sequenced alongside our *Vexillum* samples. The reads that did not map to any genome
 567 were retained for assembly. They were trimmed using Trimmomatic v0.36 (Bolger et al. 2014) with the
 568 following parameters: ILLUMINACLIP option enabled, seed mismatch threshold = 2, palindrome clip
 569 threshold = 40, simple clip threshold of 20; SLIDING WINDOW option enabled, window size = 4,
 570 quality threshold = 15; MINLEN = 36; LEADING = 3; TRAILING = 3 and assembled using Trinity v2.11
 571 (Grabherr et al. 2011) with default parameters (kmer size=25, transcript identity=0.98, minimal contig
 572 length=200. We used RSEM v1.3.1 (Li & Dewey 2011) with the Bowtie2 mapper, to produce TPM-based
 573 measures of transcript abundances, according to the most common practice (Phuong et al. 2016; Abalde et
 574 al. 2018; Fedosov et al. 2021). We did not perform TMM correction among samples because it requires
 575 generating single assembly for each species which we abandoned, because it resulted in a reduced number
 576 of reads mapped), and still does not allow for normalization among species. Same Trinity assemblies
 577 were used to evaluate completeness of the datasets based on two BUSCO datasets, the metazoan dataset
 578 (954 loci), and the Mollusca dataset (5295 loci) (Waterhouse et al. 2018). We retrieved TPM expressions
 579 levels for the complete BUSCOs extracted from each dataset, and ranged them by increasing TPM
 580 expression level. We arbitrarily denoted the TPM expression level corresponding to the 25 percentile of
 581 this distribution as the minimal confidence threshold: the predicted transcripts of this dataset with lower
 582 expression levels were discarded.

Coding DNA sequences (CDSs) were predicted from the Trinity assembly using ORFfinder (NCBI), keeping only those CDSs that comprised over 35 amino acid residues. First, they were further filtered to remove possible cross-contaminations by applying the following filter: if an CDS showed TPM expression level ≤ 0.01 relative to an identical CDS from some other specimen sequenced at the same facility, the former CDS was removed from the dataset (custom Python script PS1.py). Then, a non-redundant catalog of all remaining CDSs was built for each species, where CDSs were ranked by their TPM expression levels summed across specimens. The secreted gene products were identified as CDSs that contain a signal sequence, identified by SignalP v5.0 (Nielsen 2017) with a D-value, $D \geq 0.7$, but lack a transmembrane domain, detected by phobius v1.01 (Käll et al. 2007). To detect putative assembly errors, in the CDSs that passed this filter, we retrieved the per-base coverage data for each CDS using samtools_depth function, and a custom Python script PS2.py, and checked it to ensure that there are no abrupt shifts in the transcript coverage. The subset of CDSs that passed these filters was subjected to a sequence-based annotation by means of BLASTp against the manually curated SWISS-Prot database (Bairoch & Apweiler 2000), and the structure-based annotation using HMMER v3.2.1 (Finn et al. 2011) against the database of Hidden Markov Models, HMMs derived from Pfam (Mistry et al. 2021).

Transcripts de novo annotation

Because there are no genomic resources available for *Vexillum* or any closely related lineage of Neogastropoda, we expect that only a subset of *Vexillum* venom components can be revealed by the reference based annotation (Fedosov et al. 2021). Therefore, we first performed CDSs clustering, and then those clusters that showed high expression in either sg or gL were annotated. First, we combined four CDSs catalogs corresponding to the secreted gene products of our four species in single file, and then clustered them using two alternative approaches. Because signal sequence is highly conserved in *Conus* toxins, the classification of conotoxin gene superfamilies relies on its identity (Puillandre et al. 2012), and so a conotoxin can be assigned to a gene superfamily based on the signal sequence matching. Most conotoxin gene superfamilies show 0.55 – 0.7 Percent identity (PID) of the signal sequence (Kaas et al. 2012). We used CD-Hit (Fu et al. 2012) with two values of PID, 0.6 and 0.65 to generate two alternative sets of clusters for our ORFs. However, the algorithm of CD-Hit tends to neglect similarity of longer sequences, and therefore, they may end up in different clusters despite sharing a highly identical region. As an alternative approach, we inferred orthogroups based on the whole CDS comparisons by Orthofinder2 (Emms & Kelly 2019). Because subsequent annotation required laborious manual curation, we only focused on the highly expressed transcript clusters. We built a reduced dataset, which contained all sequence of a given SS- cluster, or of a given orthogroup if at least one of the CDSs in this orthogroup/SS-cluster had a TPM expression value exceeding 200 (custom Python script PS3.py). This reduced dataset included 3308 CDSs, representing 1056 orthogroups and 623 signal sequence based clusters (PID=0.65). This dataset was manually curated to establish optimal cluster breakdown based on i) signal sequence identity, ii) orthogroup inference and iii) available reference-based annotation. When several alternative cluster breakdowns were suggested, whole precursor alignments were built and examined to identify the best breakdown. After the removal of truncated CDSs and orthogroups / SS-clusters of single-CDS, the final dataset included 2187 CDSs allocated to 235 putative toxins clusters. Further annotation was performed for the 146 clusters comprising 1,580 CDSs (of them 1341 complete) that either were classified as conotoxins by HMMER, or had not returned any hits in the reference based annotation.

Each cluster was assigned a code based on its summed expression, length of included CDSs, and degree of their sequence conservation. We arranged all 146 clusters based on the summed expression in descending order and assigned each cluster a number based on its position in this ranking (Table S1). Then we considered separately the 14 clusters comprising very short CDSs (<40aa), clusters with CDSs of intermediate length (exceeding 40aa, but less than 200 aa), and long CDSs (length exceeding 200 aa). The letters ‘s’ and ‘l’ were appended to the cluster code for clusters of ‘short’ and ‘long’ CDSs respectively. Letter ‘c’ was appended to the codes of those clusters that showed high level of CDS sequence conservation (length variation < 1% of the average complete CDS length, and proportion of variable sites in the aa alignment <10%). Finally, a few clusters that contained only 2-3 CDSs, were

denoted as ‘minor’, and letter ‘m’ was appended to their codes. Regardless of the expression, length, and degree of conservation, these 146 clusters were treated as putative toxins, and their precursor structure was analysed by Conoprec (available at <http://www.conoserver.org/index.php?page=conoprec>), to establish the domain breakdown, and to identify putative post-translational modifications (PTMs) and canonical cys-frameworks.

To generate additional support for the PTMs identified by ConoPrec, we performed a search for respective PTMs enzymes in sg and gL of *V. coccineum*, and *V. vulpecula*. We accessed all Uniprot sequences of the following enzymes identified in caenogastropod mollusks: Glutamyl-peptide cyclotransferase (PTM: N-terminal pyroglutamic acid), Vitamin K-dependent carboxylase (PTM: γ -carboxylated glutamic acid), peptidylglycine amidating monooxygenase (PTM: C-terminal amidation), and Prolyl 4-hydrolase, carrying out PTM of proline to 4-hydroxy proline, the fourth common PTM in conotoxins. Furthermore, we accessed Uniprot sequences of the two enzymes that have been shown to play important role in folding of conotoxins: protein disulfide isomerase and peptidylprolyl cys-trans isomerase (Safavi-Hemami et al. 2010). All predicted transcripts in the Trinity assemblies of *V. coccineum* and *V. vulpecula* that generated a blastx hit to any of the PTM enzymes sequences with aligned length $\geq 50\%$ of the respective database entry length, and the BLAST e-value ≤ -25 were recorded, and their expression levels were summed up.

We predicted 3D structure of mature peptide domains for a few putative toxins for which we recovered sufficient support in the proteomic data. The structure modeling was performed in the ColabFold notebook (available at https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/beta/AlphaFold2_advanced_beta.ipynb), which implements the recently released AlphaFold2 (Jumper et al. 2021). The multiple sequence alignments were built using the MMseqs2 algorithm, following by the prediction of five best spatial models, based on their mean pLDDT (Local Distance Difference Test) scores. The model with the highest LDDT-score was refined using Amber (Case et al. 2005)(available as a part of the ColabFold workflow), and then visualized in Chimera v1.15 (Pettersen et al. 2004) to infer cysteine connectivity. This model was also used for the structure-based search, which we performed using the online RUPEE protein structure search (Ayoub & Lee 2019) (available at <https://ayoubresearch.com/>) against the SCOPe database (Chandonia et al. 2019).

Phylogenetic inference

To reconstruct species tree of the four analyzed *Vexillum* species, the complete BUSCOs extracted from each transcriptomic dataset were merged to build a non-redundant catalog of BUSCOs for each species. The amino acid sequences were aligned separately for each BUSCO locus using MAFFT v7.407 (Katoh & Standley 2013), and then a concatenated matrix was built from those 426 BUSCO loci from the Mollusca dataset that were present in all four *Vexillum* species (custom Python script PS4.py). This matrix comprising a total of 126,681 aligned aa sites was passed to IQtree v1.6.9 (Nguyen et al. 2015) as a single partition for phylogenetic inference.

To reconstruct gene tree of the V027 vexitoxin cluster, the nucleotide sequences of the ten complete CDSs identified in this cluster were codon-aligned using MACSE v2 (Ranwez et al. 2018). Then we ran IQtree with 1000 ultra-fast bootstrap iterations, treating three codon positions in the alignment as three separate partitions.

Evolutionary analysis

The codon-aligned coding sequences of *Vexillum* V027 cluster were analysed using the HyPhy package for sequence evolution inference (Kosakovsky Pond et al. 2020). Three methods, Fixed Effects Likelihood (FEL), Single-Likelihood Ancestor Counting (SLAC) and Fast Unconstrained Bayesian AppRoximation (FUBAR) were applied to test for pervasive selection across the alignment. The sites under episodic diversifying selection (i.e. acting on a branch of a phylogenetic tree) were inferred by Mixed Effects Model of Evolution (MEME).

682 *Sample preparation for proteomic analysis*

683 The specimens of salivary glands and glands of Leiblein for proteomic analysis were transported frozen to
 684 the laboratory and stored at -80°C. Each sample was used for both protein and intact peptide extraction,
 685 and three replicates of each tissue / species were analyzed resulting in a total of 12 analyzed samples.
 686 First, 500 µl of lysis buffer containing 2% sodium deoxycholate (SDC) in 100mM Tris (pH 8.5)
 687 preheated at 95°C was added to each specimen. Then the specimens were fragmented with scissors, and
 688 incubated at 95°C for 10 minutes. After the samples have cooled down they were subjected to sonication
 689 by Qsonica Q55 ultrasonic homogenizer (Qsonica, Newtown, CT, USA) at 80% amplitude using five
 690 series of five one-second-duration impulses. After the homogenization, the samples were centrifuges at
 691 16000 × g for 10 min and the supernatants were transported to clean tubes. Cysteine reduction and
 692 alkylation were performed simultaneously by adding tris(2-carboxyethyl)phosphine (TCEP) up to 10 mM
 693 and chloroacetamide (CAM) up to 20 mM to the samples, following incubation at 56°C for 40 min.
 694 Meanwhile, the 10 kDa MWCO regenerated cellulose Amicon filters (Merck, Germany) were
 695 preconditioned by passing first 500 µl of 100mM Tris buffer and then the same Tris buffer containing 2%
 696 SDC trough each filter. The samples were applied to the filters and spun at 14000 × g until completely
 697 filtered. Then 200 µl of 0.5M NaCl was loaded onto each filter and spun at the same speed. Both portions
 698 of the flow-through from each sample were combined and stored for future peptide cleanup. The
 699 remaining filters containing the proteins in the upper chamber were washed twice with 500 µl of 100mM
 700 Tris buffer. Finally 200 µl of the same buffer were added to the upper chamber of each filter. The protein
 701 solutions were extensively mixed and the upper chambers were twisted upside down into new collecting
 702 tubes followed with the centrifugation of the filters to thoroughly collect the proteins from the filter
 703 membranes.

704 The resulting solutions of protein contained 100mM Tris buffer with the remnants of SDC allowing
 705 for protein measurement using BCA assay. Thirty micrograms of total protein were diluted up to 30 µl
 706 with the same Tris buffer. Then trypsin was added with the proportion of 1:50 and incubated overnight at
 707 37°C. The reaction was terminated by the addition of trifluoroacetic acid (TFA) up to 1.5% to each
 708 sample resulting in consequential precipitation of SDC.

709 *Peptide cleanup*

710 Trifluoroacetic acid (TFA) was added to all peptide samples obtained both after the filtration and trypsin
 711 digestion, up to 1.5% in order to remove SDC. Then three cycles of washing were performed. In each
 712 cycle, two volumes of ethyl acetate were added to the samples in order to dissolve the residual SDC
 713 precipitate and other unwanted contaminants. The samples were vortexed followed by quick
 714 centrifugation for 2 min at 6000 rpm (maximum speed in centrifuge BioSan Multi-spin MSV-6000,
 715 BioSan, Riga, Latvia), and the upper phase was discarded.

716 For the peptide desalting and cleanup the in-house made stage tips containing SDB-RPS membrane
 717 (Empore-3M, CDS Analytical, Oxford, PA, USA) were used. The tips were prepared according to
 718 (Rappsilber et al. 2003) with the use of 3 pieces of membrane in each tip. The samples were loaded into
 719 the tips and the tips were centrifuged at 1200 rpm (about 70 × g in the same centrifuge) until the solution
 720 has passed through the membrane. At the next step, 100 µl of 1% TFA covered by 50 µl of ethyl acetate
 721 were passed through the tips at the same speed in order to remove the remnants of SDC, and then washing
 722 was performed at the same speed with 100 µl 0.2% TFA. The peptides were eluted by passing 60 µl of
 723 70% acetonitrile (CAN) with 5% NH₄OH through the tips at the speed of 1000 rpm (about 50 × g). The
 724 peptide samples were then dried in the vacuum concentrator (Labconco, Kansas City, MO, USA).

725 *Liquid chromatography and tandem mass spectrometry (LC-MS/MS)*

For the LC-MS analysis the samples were reconstituted in 0.1% TFA and loaded to a Acclaim PepMap 100 C18 (100 μ m x 2 cm) trap column in the loading mobile phase (2% acetonitrile (ACN), 98% H₂O, 0.1% TFA) at 10 μ l/min flow and separated at 40°C on a 500 mm 75 μ m inner diameter Thermo Scientific™ Acclaim™ PepMap™ 100 C18 LC column with particle size 2 μ m. Reverse-phase chromatography was performed with an Ultimate 3000 Nano LC System (Thermo Fisher Scientific), which was coupled to the Orbitrap Q Exactive HF mass spectrometer (Thermo Fisher Scientific) via a nanoelectrospray source (Thermo Fisher Scientific). the following chromatography conditions were used for the samples that underwent trypsin digestion: Water containing 0.1% (v/v) formic acid (FA) was used as mobile phase A and ACN containing 0.1% FA (v/v), 20% (v/v) H₂O as mobile phase B. Peptides were eluted from the trap column with a linear gradient: 3–35% solution B (0.1% (v/v) formic acid, 80% (v/v) acetonitrile) for 105 min; 35–55% B for 18 min, 55–99% B for 0.1 min, 99% B during 10 min, 99–2% B for 0.1 min at a flow rate of 300 nl/min. After each gradient, the column was re-equilibrated with A for 10 min. Similar conditions were used for the samples containing intact peptides, but the total gradient time was 60 min. MS data was collected in DDA mode (TopN=15), with the following MS1 parameters: resolution 120K, scan range 350–1400, max injection time – 50 msec, AGC target – 3×10^6 . Ions were isolated with 1.2 m/z window, preferred peptide match and isotope exclusion. Dynamic exclusion was set to 30 s. MS2 fragmentation was carried out at 15K resolution with HCD collision energy set to 28, max injection time – 80 msec, AGC target – 1×10^5 . Other settings: charge exclusion - unassigned, 1, 6–8, >8.

Bioinformatic integration of the mass spec data

The non-redundant catalogs of CDSs predicted from transcriptomic data of conspecific *Vexillum* specimens were used as databases for the proteomic search. Two different databases were built for each species. First database contained only mature toxin regions (Supp.Data3) of the transcripts of interest (i.e. putative venom components) and was used for the analysis of peptidomes in the samples of intact peptides. Second database contained all the predicted CDSs of a species, where the transcripts of interest contained a recognizable pattern in their sequence identifiers; this database was used for search of the spectra obtained after the trypsin digestions of the proteins.

All the .raw files were converted to .mzML format with ThermoRawFileParser (Hulstaert et al. 2020). The search engine IdentiPy v.0.3.3.16 (Levitsky et al. 2018) was used for proteins searches of data obtained after the trypsin digestion, followed by the post-search treatment and result filtration by Scavenger v.0.2.4 (Ivanov et al. 2019). For the search, trypsin was chosen as a parameter and the number of allowed missed cleavages was set to 1. Mass accuracy for the precursor and the fragment ions were set to 10 ppm and 0.01 Da respectfully. Carbamidomethylation of Cys was set as a fixed modification, oxidation of Met, and deamidation of Gln and Asn - as variable modifications. The clusters of interest were filtered group-specifically with Scavenger according to the target-decoy strategy with 1% false-discovery rate cut-off.

The .mzML files obtained for the samples with intact peptide extraction, were subjected to *de novo* peptide sequencing with PEAKS CMD (v. 1.0). The precursor and fragment mass accuracies were set to 10 ppm and 0.01 Da respectively, and no protease was selected, as there was no digestion performed for these samples. The results were filtered to at least 80% average confidence in peptide. The resulting peptides were mapped to the mature toxin sequences database, accounting for the identical masses of Leu and Ile.

767 **References**

- 768 Abalde S, Tenorio MJ, Afonso CML, Zardoya R. 2018. Conotoxin Diversity in *Chelyconus*
769 *ermineus* (Born, 1778) and the Convergent Origin of Piscivory in the Atlantic and Indo-
770 Pacific Cones. *Genome Biol Evol.* 10: 2643–2662.
- 771 Abdelkrim J et al. 2018. Exon-capture based phylogeny and diversification of the venomous
772 gastropods (Neogastropoda, Conoidea). *Mol Biol Evol.* 35:2355–2374.
- 773 Ayoub R, Lee Y. 2019. RUPEE: A fast and accurate purely geometric protein structure search.
774 *PLOS ONE.*
- 775 Bairoch A, Apweiler R. 2000. The SWISS-PROT protein sequence database and its supplement
776 TrEMBL in 2000. *Nucleic Acids Res.* 28:45–48.
- 777 Barghi N, Concepcion GP, Olivera BM, Lluisma AO. 2015. Comparison of the Venom Peptides
778 and Their Expression in Closely Related *Conus* Species: Insights into Adaptive Post-
779 speciation Evolution of *Conus* Exogenomes. *Genome Biol Evol.* 7:1797–1814.
- 780 Barua A, Mikheyev AS. 2021. An ancient, conserved gene regulatory network led to the rise of
781 oral venom systems. *Proc Natl Acad Sci USA.* 118:e2021311118.
- 782 Barua A, Mikheyev AS. 2019. Many Options, Few Solutions: Over 60 My Snakes Converged on
783 a Few Optimal Venom Formulations. *Mol Biol Evol.* 36:1964–1974..
- 784 Biggs JS, Olivera BM, Kantor Y. 2008. Alpha-conopeptides specifically expressed in the
785 salivary gland of *Conus pulicarius*. *Toxicon.* 52:101–105.
- 786 Bjørn-Yoshimoto WE et al. 2020. Curses or Cures: A Review of the Numerous Benefits Versus
787 the Biosecurity Concerns of Conotoxin Research. *Biomedicines.* 8:235.
- 788 Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence
789 data. *Bioinformatics.* 30:2114–2120.
- 790 Case DA et al. 2005. The Amber Biomolecular Simulation Programs. *J Comput Chem.* 26:1668–
791 1688.
- 792 Casewell NR et al. 2017. The Evolution of Fangs, Venom, and Mimicry Systems in Blenny
793 Fishes. *Curr Biol.* 27:1184–1191.
- 794 Casewell NR, Wüster W, Vonk FJ, Harrison RA, Fry BG. 2013. Complex cocktails: the
795 evolutionary novelty of venoms. *Trends Ecol Evol.* 28:219–229.
- 796 Castañeda O et al. 1995. Characterization of a potassium channel toxin from the Caribbean Sea
797 anemone *Stichodactyla helianthus*. *Toxicon.* 33:603–613.

798 Chandonia J-M, Fox NK, Brenner SE. 2019. SCOPe: classification of large macromolecular
799 structures in the structural classification of proteins—extended database. *Nucleic Acids*
800 *Res.* 47:D475–D481.

801 Chang D, Duda TF. 2012. Extensive and Continuous Duplication Facilitates Rapid Evolution
802 and Diversification of Gene Families. *Mol Biol Evol.* 29: 2019–2029.

803 Chi V et al. 2012. Development of a sea anemone toxin as an immunomodulator for therapy of
804 autoimmune diseases. *Toxicon.* 59:529–546.

805 Dutertre S et al. 2014. Evolution of separate predation- and defence-evoked venoms in
806 carnivorous cone snails. *Nat commun.* 3521:1–9.

807 Emms DM, Kelly S. 2019. OrthoFinder: phylogenetic orthology inference for comparative
808 genomics. *Genome Biol.* 20:238.

809 Fassio G et al. 2019. Venom Diversity and Evolution in the Most Divergent Cone Snail Genus
810 *Profundiconus*. *Toxins.* 11:623.

811 Fedosov A, Zaharias P, Puillandre N. 2021. A phylogeny-aware approach reveals unexpected
812 venom components in divergent lineages of cone snails. *Proc. R. Soc. B.* 288:20211017.

813 Fedosov AE et al. 2019. Mapping the missing branch on the Neogastropoda tree of life:
814 molecular phylogeny of marginelliform gastropods. *J Moll Stud.* 58: 439–451.

815 Fedosov AE, Kantor YI. 2010. Evolution of carnivorous gastropods of the family Costellariidae
816 (Neogastropoda) in the framework of molecular phylogeny. *Ruthenica.* 20: 117–139.

817 Fedosov AE, Puillandre N, Herrmann M, Dgebuadze P, Bouchet P. 2017. Phylogeny,
818 systematics and evolution of the family Costellariidae (Gastropoda: Neogastropoda). *Zool*
819 *J Linn Soc-Lond.* 179: 541–626.

820 Finn RD, Clements J, Eddy SR. 2011. HMMER web server: interactive sequence similarity
821 searching. *Nucleic Acids Res.* 39:W29–W37.

822 Fu L, Niu B, Zhu Z, Wu S, Li W. 2012. CD-HIT: accelerated for clustering the next-generation
823 sequencing data. *Bioinformatics.* 28:3150–3152.

824 Gerdol M et al. 2019. A Recurrent Motif: Diversity and Evolution of ShKT Domain Containing
825 Proteins in the Vampire Snail *Cumia reticulata*. *Toxins.* 11:106.

826 Grabherr MG et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a
827 reference genome. *Nat Biotechnol.* 29:644–652.

828 Hargreaves AD, Swain MT, Hegarty MJ, Logan DW, Mulley JF. 2014. Restriction and
829 Recruitment—Gene Duplication and the Origin and Evolution of Snake Venom Toxins.
830 *Genome Biol Evol.* 6:2088–2095.

831 Hu H, Bandyopadhyay PK, Olivera BM, Yandell M. 2012. Elucidation of the molecular
832 envenomation strategy of the cone snail *Conus geographus* through transcriptome
833 sequencing of its venom duct. *BMC Genomics.* 13:284.

834 Hulstaert N et al. 2020. ThermoRawFileParser: Modular, Scalable, and Cross-Platform RAW
835 File Conversion. *J Proteome Res.* 19:537–542.

836 Imperial JS et al. Using Chemistry to Reconstruct Evolution: On the Origins of Fish-hunting in
837 Venomous Cone Snails.

838 Ivanov MV, Levitsky LI, Bubis JA, Gorshkov MV. 2019. Scavager: A Versatile Postsearch
839 Validation Algorithm for Shotgun Proteomics Based on Gradient Boosting. *Proteomics.*
840 19:1800280.

841 Jumper J et al. 2021. Highly accurate protein structure prediction with AlphaFold. *Nature.*
842 596:583–589.

843 Kaas Q, Yu R, Jin A-H, Dutertre S, Craik DJ. 2012. ConoServer: updated content, knowledge,
844 and discovery tools in the conopeptide database. *Nucleic Acids Res.* 40:D325–D330.

845 Käll L, Krogh A, Sonnhammer ELL. 2007. Advantages of combined transmembrane topology
846 and signal peptide prediction—the Phobius web server. *Nucleic Acids Res.* 35:W429–
847 W432.

848 Kalman K et al. 1998. ShK-Dap22, a potent Kv1.3-specific immunosuppressive polypeptide. *J*
849 *Biol Chem.* 273:32697–32707.

850 Kantor YI. 2002. Morphological prerequisites for understanding neogastropod phylogeny.
851 *Bollettino Malacologico.* Suppl. 4:161–174.

852 Kantor YI, Fedosov AE. 2009. Morphology and development of the valve of Leiblein: Possible
853 evidence for paraphyly of the Neogastropoda. *Nautilus.* 123:73–82.

854 Katoh K, Standley DM. 2013. MAFFT Multiple Sequence Alignment Software Version 7:
855 Improvements in Performance and Usability. *Mol Biol Evol.* 30:772–780.

856 Kohn A. 2018. *Conus* Envenomation of Humans: In Fact and Fiction. *Toxins.* 11:10.

857 Kohn AJ. 1990. Tempo and mode of evolution in Conidae. *Malacologia.* 32:55–67.

858 Kosakovsky Pond SL et al. 2020. HyPhy 2.5—A Customizable Platform for Evolutionary
859 Hypothesis Testing Using Phylogenies. *Mol Biol Evol* 37:295–299.

860 L DCM et al. 2014. α -conotoxin RgIA protects against the development of nerve injury-induced
861 chronic pain and prevents both neuronal and glial derangement. *Pain*. 155.

862 Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods*.
863 9:357–359.

864 Lavergne V et al. 2015. Optimized deep-targeted proteotranscriptomic profiling reveals
865 unexplored Conus toxin diversity and novel cysteine frameworks. *Proc Natl Acad Sci USA*.
866 112:E3782–E3791.

867 Lebbe EKM, Tytgat J. 2016. In the picture: disulfide-poor conopeptides, a class of
868 pharmacologically interesting compounds. *J Venom Anim Toxins Incl Trop Dis*. 22:30.

869 Levitsky LI et al. 2018. IdentiPy: An Extensible Search Engine for Protein Identification in
870 Shotgun Proteomics. *J Proteome Res*. 17:2249–2255.

871 Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or
872 without a reference genome. 16.

873 Lu A et al. 2020. Transcriptomic Profiling Reveals Extraordinary Diversity of Venom Peptides
874 in Unexplored Predatory Gastropods of the Genus *Clavus*. *Genome Biol Evol*. 12:684–700.

875 Maes VO, Raeihle D. 1975. Systematics and biology of *Thala floridana* (Gastropoda:
876 Vexillidae). *Malacologia*. 15:43–67.

877 Mistry J et al. 2021. Pfam: The protein families database in 2021. *Nucleic Acids Res*. 49:D412–
878 D419.

879 Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: A Fast and Effective
880 Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Mol Biol Evol*.
881 32:268–274.

882 Nielsen H. 2017. Predicting Secretory Proteins with SignalP. In: Protein Function Prediction:
883 Methods and Protocols. Kihara, D, editor. Methods in Molecular Biology Springer: New
884 York, NY pp. 59–73.

885 Olivera BM, Seger J, Horvath MP, Fedosov AE. 2015. Prey-capture Strategies of Fish-hunting
886 Cone Snails: Behavior, Neurobiology and Evolution. *Brain Behav Evol*. 86:58–74.

887 Olivera BM, Showers Corneli P, Watkins M, Fedosov A. 2014. Biodiversity of Cone Snails and
888 Other Venomous Marine Gastropods: Evolutionary Success Through Neuropharmacology.
889 *Annu rev Anim Biosci*. 2:487–513.

890 Pallaghy PK, Nielsen KJ, Craik DJ, Norton RS. 1994. A common structural motif incorporating
891 a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. *Protein*
892 *Sci.* 3:1833–1839.

893 Pennington MW et al. 1995. Chemical synthesis and characterization of ShK toxin: a potent
894 potassium channel inhibitor from a sea anemone. *Int J Pept Protein Res.* 46:354–358.

895 Pettersen EF et al. 2004. UCSF Chimera--a visualization system for exploratory research and
896 analysis. *J Comput Chem.* 25:1605–1612.

897 Phuong MA, Mahardika GN, Alfaro ME. 2016. Dietary breadth is positively correlated with
898 venom complexity in cone snails. *BMC Genomics.* 17:1–15.

899 Ponder WF. 1973. The origin and evolution of the Neogastropoda. *Malacologia.* 12:295–338.

900 Ponte G, Modica MV. 2017. Salivary glands in predatory mollusks: Evolutionary considerations.
901 *Front physiol.* 8:1–8.

902 Prashanth JR, Brust A, Alewood PF, Dutertre S, Lewis RJ. 2014. Cone snail venomics: from
903 novel biology to novel therapeutics. *Future Med Chem.* 6:1659–75.

904 Puillandre N, Fedosov AE, Kantor YI. 2016. Systematics and Evolution of the Conoidea. In:
905 Evolution of Venomous Animals and Their Toxins. Gopalakrishnakone, P, editor. Springer
906 pp. 1–32.

907 Puillandre N, Koua D, Favreau P, Olivera BM, Stocklin R. 2012. Molecular phylogeny,
908 classification and evolution of conopeptides. *J Mol Evol.* 74:297–309.

909 Ranwez V, Douzery EJP, Cambon C, Chantret N, Delsuc F. 2018. MACSE v2: Toolkit for the
910 Alignment of Coding Sequences Accounting for Frameshifts and Stop Codons. *Mol Biol*
911 *Evol.* 35:2582–2584.

912 Rappsilber J, Ishihama Y, Mann M. 2003. Stop and Go Extraction Tips for Matrix-Assisted
913 Laser Desorption/Ionization, Nanoelectrospray, and LC/MS Sample Pretreatment in
914 Proteomics. *Anal Chem.* 75:663–670.

915 Robinson SD et al. 2017. Hormone-like peptides in the venoms of marine cone snails. *Gen Comp*
916 *Endocr.* 244:11–18.

917 Robinson SD, Norton RS. 2014. Conotoxin Gene Superfamilies. *Mar Drugs.* 12:6058–6101.

918 Safavi-Hemami H, Brogan SE, Olivera BM. 2019. Pain therapeutics from cone snail venoms:
919 From Ziconotide to novel non-opioid pathways. *J Proteomics.* 190:12–20.

920 Safavi-Hemami H, Bulaj G, Olivera BM, Williamson NA, Purcell AW. 2010. Identification of
921 *Conus* Peptidylprolyl Cis-Trans Isomerases (PPIases) and Assessment of Their Role in the
922 Oxidative Folding of Conotoxins. *J Biol Chem.* 285:12735–12746.

923 Schendel V, Rash LD, Jenner RA, Undheim EAB. 2019. The Diversity of Venom: The
924 Importance of Behavior and Venom System Morphology in Understanding Its Ecology and
925 Evolution. 22.

926 Shah AA, Khan YD. 2020. Identification of 4-carboxyglutamate residue sites based on position
927 based statistical feature and multiple classification. *Sci Rep.* 10:16913.

928 Suzuki N et al. 2008. Structures of pseudochetoxin and pseudocin, two snake-venom cysteine-
929 rich secretory proteins that target cyclic nucleotide-gated ion channels: implications for
930 movement of the C-terminal cysteine-rich domain. *Acta Crystallogr D Biol Crystallogr.*
931 64:1034–1042.

932 Tarcha EJ et al. 2017. Safety and pharmacodynamics of dalazatide, a Kv1.3 channel inhibitor, in
933 the treatment of plaque psoriasis: A randomized phase 1b trial. *PLoS One.* 12:e0180762.

934 Taylor JD, Morris NJ, Taylor CN. 1980. Food specialization and the evolution of predatory
935 prosobranch gastropods. *Palaeontology.* 23:375–409.

936 Terlau H, Olivera BM. 2004. *Conus* Venoms: A Rich Source of Novel Ion Channel-Targeted
937 Peptides. *Physiol rev.* 84:41–68.

938 Wang J et al. 2005. Blocking effect and crystal structure of natrin toxin, a cysteine-rich secretory
939 protein from *Naja atra* venom that targets the BKCa channel. *Biochemistry.* 44:10145–
940 10152.

941 Waterhouse RM et al. 2018. BUSCO Applications from Quality Assessments to Gene Prediction
942 and Phylogenomics. *Mol Biol Evol.* 35:543–548.

943 Wingett SW, Andrews S. 2018. FastQ Screen: A tool for multi-genome mapping and quality
944 control. doi: 10.12688/f1000research.15931.2.

945 Zancolli G, Casewell NR. 2020. Venom Systems as Models for Studying the Origin and
946 Regulation of Evolutionary Novelties. *Mol Biol Evol.* 37:2777–2790. d

947 Zhang K et al. 2011. An essential role of the cysteine-rich domain of FZD4 in Norrin/Wnt
948 signaling and familial exudative vitreoretinopathy. *J Biol Chem.* 286:10210–10215.

949