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Comparative transcriptomics of the venoms of continental and insular radiations of West African cones

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The transcriptomes of the venom glands of 13 closely related species of vermivorous cones endemic to West Africa from genera *Africonus* and *Varioconus* were sequenced and venom repertoires compared within a phylogenetic framework using one *Kalloconus* species as outgroup. The total number of conotoxin precursors per species varied between 108 and 221. Individuals of the same species shared about one-fourth of the total conotoxin precursors. The number of common sequences was drastically reduced in the pairwise comparisons between closely related species, and the phylogenetical signal was totally eroded at the inter-generic level (no sequence was identified as shared derived), due to the intrinsic high variability of these secreted peptides. A common set of four conotoxin precursor superfamilies (T, O1, O2 and M) was expanded in all studied cone species, and thus, they are considered the basic venom toolkit for hunting and defense in the West African vermivorous cone snails. Maximum-likelihood ancestral character reconstructions inferred shared conotoxin precursors preferentially at internal nodes close to the tips of the phylogeny (between individuals and between closely related species) as well as in the common ancestor of *Varioconus*. Besides the common toolkit, the two genera showed significantly distinct catalogues of conotoxin precursors in terms of type of superfamilies present and the abundance of members per superfamily, but had similar relative expression levels indicating functional convergence. Differential expression comparisons between vermivorous and piscivorous cones highlighted the importance of the A and S superfamilies for fish hunting and defense.

1. Introduction

Cones (Gastropoda: Conidae) are marine venomous predators that actively hunt on worms, snails and fish [1]. Their venom is a cocktail constituted by hundreds of peptides named conotoxins, as well as by hormones and by other proteins that participate in the synthesis or enhance the activity of the venom [2,3]. Once inside the prey, conotoxins interact with ion channels and neurotransmitter receptors triggering different physiological responses, from sedation to tetanic paralysis [4]. Conotoxin precursors typically present a three domain structure, consisting of signal, pro-peptide and mature (i.e. the functional toxin after processing of the precursor) regions [5]. The signal region is conserved, and it is used to classify the peptides into different 'superfamilies' [4]. The composition of the venom is highly variable among species, specimens and even within the same individual depending on its physiological status or ecological interactions [6–13].

Most cone snail venomic studies have been driven preferentially by the pharmacological potential of conotoxins and were limited to the purification of

mature peptides and the identification of their function. Thus, they lack the wider evolutionary perspective already applied in the study of other venomous animals [14–16]. Comparing venom cocktails from different cone species within a phylogenetic framework should provide insights on how the rich conotoxin diversity was generated [12,17], to what extent distinct venom repertoires are adapted to different diet specializations [10,18,19], and which are the functional constraints and levels of convergence imposed by this coevolutionary arms race system [8,20], among others.

As in other venomous animals [16,21], dietary breadth has been proposed to be a main factor triggering venom evolution in cones [18,19,22]. Since hunting performance relies on venom specificity, shifts in diet could trigger changes in venom composition [10,23,24] and in general, species with more generalized diets would tend to have more complex venoms [19,22]. Moreover, instances of functional convergence have been shown in the venom cocktails of Atlantic and Indo-Pacific piscivorous cones [8]. Another level of evolutionary complexity comes from the capacity of cones to modulate the composition of their venom depending on its final use, whether to subdue preys or defend themselves against predators [12,25].

The above-mentioned studies explored general venom evolutionary trends at the family (Conidae) level by comparing distantly related lineages. A few studies compared venom cocktails from pairs of species within the same genus but lacked an evolutionary perspective (e.g. [7]). Here, we analyse venom evolution within two radiations of closely related cone species inhabiting West Africa [26,27]: one comprising cones endemic to the Cabo Verde archipelago, ascribed to genus *Africonus*; the other including cones endemic to Senegal (plus one closely related species inhabiting Canary Islands), recently ascribed to genus *Varioconus* [28]. Importantly, robust phylogenies based on mitogenomes are available for both clades providing the necessary framework for evolutionary studies [26,27]. The clade of cones endemic to Cabo Verde diversified about 9 Mya into four main lineages and at least 40 endemic species [26]. The clade of cones endemic to Senegal and Canary Islands diversified about 6 Mya into three main lineages and at least 13 endemic species [27]. All species in both clades are vermivorous; *Africonus* species show little apparent differences in radular tooth morphology whereas the three clades of *Varioconus* from Senegal and Canary Islands have each distinct radular teeth, suggesting subtle diet specializations [27]. No study has analysed the venom transcriptomes of these endemic cones.

Here, we sequenced the venom gland transcriptomes from 13 species belonging to genera *Africonus* and *Varioconus*, as well as one from *Kalloconus trochulus*, which was used as outgroup. We aimed to (i) describe venom compositions in terms of the presence, member diversity and relative expression levels of the conotoxin precursor superfamilies; (ii) assess the levels of divergence in venom composition at different hierarchical (taxonomic) levels and discern between shared-derived peptides and potential cases of functional convergence; (iii) determine whether there could be instances of differential expression between the two genera as footprint of adaptation; and (iv) compare differential conotoxin expression between these vermivorous species and the piscivorous species *Chelyconus ermineus* [8] and *Pionoconus magus* [29] from the Atlantic and the Indo-Pacific oceans, respectively, to further understand the connections between venom evolution to diet specialization and defense.

2. Material and methods

(a) Taxon sampling

Taxon selection was aimed at having at least one representative per main lineage of the two genera plus a close outgroup [26,27]. The complete list of specimens, species, sampling localities and museum vouchers is provided in table 1. Phylogenetic relationships based on mitogenomes are depicted in electronic supplementary material, figure S1. In order to assess intraspecific variability, for the genus *Varioconus*, we studied two specimens of *Varioconus mercator* (V_1258 and V_1302). These individuals showed distinct shell phenotypes and in fact, V_1258 could be assigned to the recently described species, *Varioconus stimpsonorum* [30]. However, its mitogenome sequence divergence to *V. mercator* is exactly at the threshold used to delimit species in West African cones [27]. Moreover, the results here presented in terms of venom composition (see below) strongly suggest that *V. stimpsonorum* should be considered a synonym of *V. mercator* [28], and hence, the specimen V_1258 was treated as *V. mercator* herein. In the case of *Africonus*, we included two specimens of *Africonus maioensis* (A_0055 and A_0039; representing two different shell phenotypes formerly classified as distinct species but now synonymized; [27]). All the specimens were adults and were dissected in a resting stage to remove the venom duct, which was preserved in RNAlater (Invitrogen, Life technologies).

(b) RNA extraction and sequencing

RNA extraction and sequencing were performed as in [8]. Briefly, each venom duct was incubated with 500 µl of TRIzol LS Reagent (Invitrogen, Life Technologies) and grinded with ceramic beads in a Praecellys Evolution homogenizer. Total RNA was purified using the Direct-Zol RNA Miniprep kit (Zymo Research, Irvine) following the manufacturer's instructions. Dual-indexed cDNA libraries were constructed for each sample using the TruSeq RNA library Prep kit v2 (Illumina, San Diego) at Sistemas Genómicos (Valencia, Spain) following the manufacturer's instructions. After the quality of the libraries was checked, they were pooled and split into several runs of paired-end sequencing (2 × 100 bp) in an Illumina HiSeq2500 (each sample divided into two flow cells to avoid sequencing biases) following the standard procedures at Sistemas Genómicos (Valencia, Spain).

(c) Transcriptome assembly and conotoxin identification

The raw reads corresponding to the different individuals were sorted using the library indices, which were removed using Cutadapt v.1.3 [31]. Raw read quality was checked with FastQC v.0.10.1 (www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the assembly was performed using Trinity v.2.6.6 [32] with default settings (minimum contig length = 200 bp, sequence identity threshold = 0.95) and the trimmomatic option active with default parameters. The raw reads of all transcriptomes are available at the SRA database (table 1).

Conotoxin precursors, hormones and associated venom proteins available in GenBank release 222, Uniprot release 2017_09, and ConoServer release 30/10/2017 were downloaded 30 October 2017 and concatenated into a single fasta file. Duplicated sequences were removed, and the resulting file was formatted to create the custom reference database using BLAST+ [33].

Proteins of interest in the assembled transcriptomes were identified using BLASTX over the custom reference database (e-value: 1×10^{-5}). The amino acid sequences were manually inspected and those considered as false positives or assembly artefacts (showing internal stop codons and chimeras), those that were duplicated or highly truncated (missing greater than 55% of the estimated length of the reference protein), and those showing low coverage values were discarded. We implemented an extra

Table 1. Specimens analysed in this study and main statistics of Illumina sequencing and assembly.

ID	species	country	locality/island	voucher MNCN	SRA accesion	sequencing date (DD/MM/YYYY)	number reads	% clean reads	number contigs	number BLAST hits	number proteins	number conotoxins
A_0885	*Africonus antoniomonteiroi*	Cabo Verde	Pedra Lume, Sal	15.05/79794	SRR11807494	21-12-2016	26 026 957	100	83 565	731	214	159
A_0520	*Africonus boavistensis*	Cabo Verde	Ervatao, Boa Vista	15.05/80413	SRR11807497	21-12-2016	26 715 260	100	39 935	797	199	168
A_0855	*Africonus cuneolus*	Cabo Verde	Fontona, Sal	15.05/79764	SRR11807496	13-05-2015	14 771 346	99.48	46 983	800	195	154
A_0048	*Africonus galeao*	Cabo Verde	Navío Quebrado, Maio	15.05/78673	SRR11807500	21-12-2016	28 109 709	100	50 811	803	180	151
A_1387	*Africonus grahami*	Cabo Verde	Calhau, São Vicente	15.05/78549	SRR11807507	21-12-2016	22 718 525	100	51 601	850	183	156
A_0025	*Africonus infinitus*	Cabo Verde	Ponta do Pau Seco, Maio	15.05/78650	SRR11807493	13-03-2014	35 854 397	98.52	76 339	1091	203	167
A_0039	*Africonus maioensis*	Cabo Verde	Praia Santana, Maio	15.05/78664	SRR11807501	28-10-2013	52 523 501	100	78 886	783	237	188
A_0055	*Africonus maioensis*	Cabo Verde	Navío Quebrado, Maio	15.05/78680	SRR11807499	28-10-2013	44 748 977	100	105 099	850	268	214
A_0875	*Africonus miruchae*	Cabo Verde	Terrinha Fina, Sal	15.05/79784	SRR11807495	21-12-2016	24 097 307	100	60 347	615	176	136
A_0031	*Africonus raulsilvai*	Cabo Verde	Praia da Soca, Maio	15.05/78656	SRR11807492	28-10-2013	56 718 528	100	99 699	1249	220	183
A_0239	*Africonus verdensis*	Cabo Verde	Tarrafal, Santiago	15.05/78864	SRR11807498	28-10-2013	40 237 424	100	77 906	1266	239	197
V_CG13	*Varioconus guanche*	Spain	Playa del Cable, Lanzarote	—	SRR11807502	08-03-2016	29 973 740	100	85 276	815	245	195
V_1258	*Varioconus mercator*	Senegal	Almadies	15.05/78419	SRR11807505	08-03-2016	28 883 175	100	66 684	631	205	167
V_1302	*Varioconus mercator*	Senegal	Ndayane	15.05/78463	SRR11807503	08-03-2016	28 392 465	100	75 406	783	261	221
V_1278	*Varioconus reticulatus*	Senegal	Ngor	15.05/78439	SRR11807504	21-12-2016	24 263 358	100	50 358	479	142	108
K_0010	*Kalloconus trochulus*	Cabo Verde	Ponta do Pau Seco, Maio	15.05/78635	SRR11807506	13-05-2015	75 347 025	96.19	69 688	1114	179	141

curation step consisting on TBLASTX searches over the nr database in GenBank to discard wrong open reading frame (ORF) assignations.

The retained sequences constituted our working list of conotoxin precursors, hormones and associated venom proteins (electronic supplementary material, file S1; table S1). The three domain structure and cysteine frameworks of conotoxin precursor alignments were inferred using Conoprec [5]. Proteins were assigned to a given superfamily by comparison with best-hit results using BLASTP searches against GenBank, and in the case of the conotoxin precursors, taking into consideration the percentage of identity in the signal region using a general threshold of 70% [4]. We further checked the correct identification of all conotoxin precursor superfamilies by aligning all the signal regions and building a neighbour-joining dendrogram (electronic supplementary material, figure S2) based on uncorrected *p* distances using ClustalW [34]. Within each superfamily, sequences were assigned to different groups of paralogy based on the sequence divergence at the pro-peptide region, the presence of different cysteine frameworks in the mature peptide and the recovery of clades in the reconstructed dendrogram. Those sequences that did not match any previously reported conotoxin precursor superfamily were classified into unassigned superfamilies and described here.

(d) Comparative analyses of venom composition

The conotoxin precursors of each species were pairwise compared. All sequences that were common to two or more species were mapped onto the reconstructed phylogeny (electronic supplementary material, figure S1) and analysed using maximum-likelihood ancestral character reconstruction as implemented in BayesTraits v. 2.0.2 (www.evolution.rdg.ac.uk; [35]). The MultiState model was used, and 10 attempts per tree were conducted.

In order to infer venom composition similarities between species and genera, we performed (i) a multiple correspondence analysis (MCA) on the presence or absence of superfamilies (binary data); (ii) a principal component analysis (PCA) on the relative member abundance by estimating the percentage of the different superfamily members; and (iii) a PCA on the relative expression level of the different superfamilies by calculating transcripts per million (TPMs; see expression analyses below). In addition, we ran a PCA comparing relative superfamily member abundances in vermivorous *Africonus* and *Varioconus* species against those in piscivorous species *C. ermineus* and *P. magus* from the Atlantic and Indo-Pacific oceans, respectively. Both MCA and PCA were performed using PAST 4.01 (https://folk.uio.no/ohammer/past/; [36]). Data were not standardized in the analyses (i.e. the variance-covariance method was used).

(e) Expression analyses

Relative expression levels for each individual were calculated by mapping the raw reads to the nucleotide sequence of each conotoxin precursor using Bowtie 2 [37], and the values were transformed to TPM estimates using RSEM [38] as implemented in Trinity v.2.6.6 [32]. We run the EBSeq software [39] to estimate for each superfamily the posterior probability of being differentially expressed (PPDE) between *Varioconus* and *Africonus*, using all the specimens of each genera as biological replicates. We considered as differentially expressed all those conotoxin precursor superfamilies with a PPDE greater than 0.95 and with a fold change above 32 (calculated as $\log_2$ RealFC ≥ 5). The same type of analysis was performed to identify those superfamilies differentially expressed in the comparison between vermivory (using the 15 specimens of West Africa as replicates) and piscivory (using the three individuals of *C. ermineus* [8] and the three individuals of *P. magus* [29]). The Shapiro–Wilk test rejected the normality of the data, so a Kruskal–Wallis test was run in R [40] over those superfamilies identified as differentially expressed to confirm these results taking variance among replicates into consideration.

3. Results

(a) Venom cataloguing of West African cones

The transcriptomes of the venom glands of 16 individuals corresponding to 14 species of genera *Africonus*, *Varioconus* and *Kalloconus* were assembled. The main statistics associated with the sequencing and assembly procedures are summarized in table 1. Overall, 2254 unique conotoxin precursor transcripts were identified (electronic supplementary material, table S1). The species with the highest and lowest number of conotoxin precursors were *V. mercator* (V_1302, 221) and *V. reticulatus* (V_1278, 108), respectively (table 1; figure 1). Conotoxin precursors were classified into 61 known superfamilies and 141 groups of paralogy taking into consideration sequence divergences in the signal and pro-peptide regions and the clades recovered in the reconstructed dendrogram (electronic supplementary material, figure S2). A total of 86 precursors could not be assigned to any known conotoxin superfamily and were grouped into seven new unassigned superfamilies (their signal sequences, cysteine frameworks and best BLAST-P hits are reported in electronic supplementary material, table S2). Several conotoxin precursor superfamilies previously reported as valid such as R, W, Z [41] and Cerm_17 [8], among others, were found to be fragments of other proteins once the right ORFs were identified using TBLASTX (electronic supplementary material, file S2).

The diversity of expanded conotoxin precursor superfamilies (i.e. those with five or more members) is represented in figure 1. These expanded conotoxin precursor superfamilies were A, Conantokin F, Con-ikot-ikot, Conkunitzin, H, I1, I4, M, O1, O2, O3, Q, T, U, Cerm_03, Cerm_08, Cerm_11 and Tand_01. The remaining 50 conotoxin precursor superfamilies were considered of minor diversity. The species *A. maioensis* (A_0055), *A. verdensis* (A_0239), *V. guanche* (V_CG13) and *V. mercator* (V_1302) presented the highest diversity of expanded superfamilies whereas *A. galeao* (A_0048) and *V. reticulatus* (V_1278) the lowest (figure 1). The O1, O2, T and M superfamilies had the highest number of members and were present in all individuals (figure 1). The Conkunitzin and Cerm_03 superfamilies also showed high member diversity but were missing in *A. verdensis* (A_0239) and in *A. cuneolus* (A_0855) plus *V. reticulatus* (V_1278), respectively (figure 1). Remarkably, different individuals of the same species could have different expansion patterns. For example, within *V. mercator,* V_1302 had expanded the H, I4 and Con-ikot-ikot superfamilies, whereas V_1258 showed expansion of the Q superfamily (figure 1).

The 16 studied individuals added up to 80 hormone sequences, which were classified into 10 families: Conopressin (with more than five members in *V. guanche*, V_CG13), Conorfamide, Insulin-related peptides 1–5, Prohormone-4a and b, Thyrostimulin hormone alpha, and Thyrostimulin hormone beta 5 (electronic supplementary material, file S1; table S1). Insulin-related peptide 5 was only present in *K. trochulus* (K_0010); Prohormone-4b and Thyrostimulin hormone alpha were only found in *Africonus antoniomonteiroi* (A_0885). In addition, 326 transcripts were assigned to 15 protein families of various functions, likely associated with venom production. Among these, the Protein Disulfide Isomerase, Conodipine and Ferritin were the most diverse. Interestingly, we identified

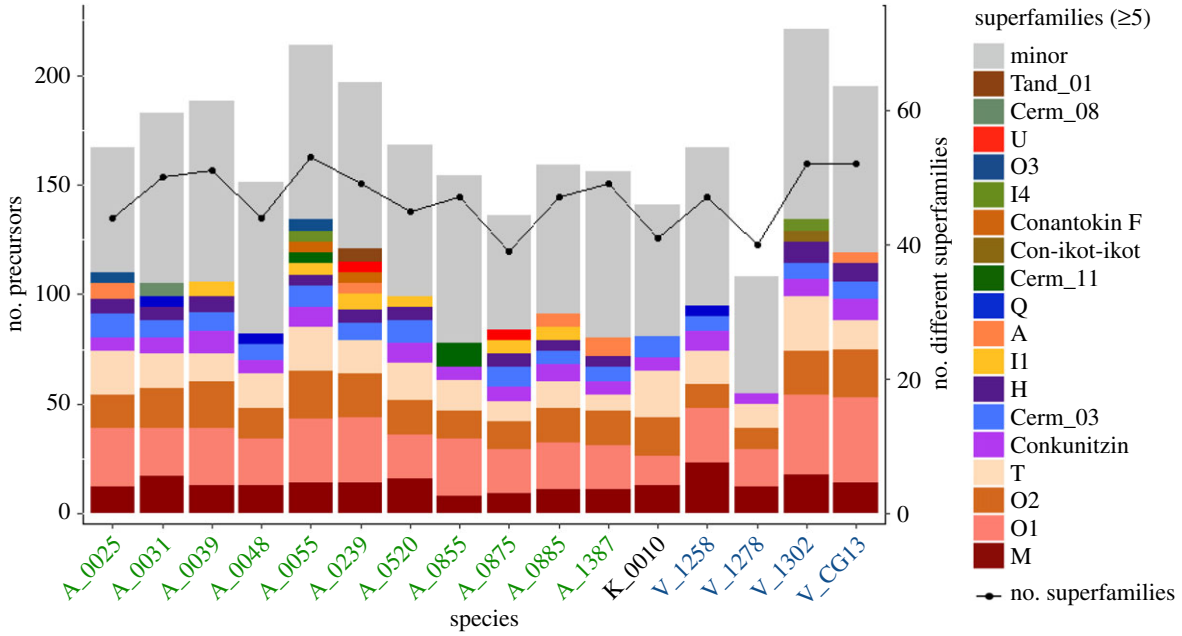


Figure 1. Venom compositions of the 16 studied specimens. The bars represent the total number of conotoxin precursors. The proportion of superfamilies with five or more members is shown in colours. The dotted line represents the number of different superfamilies identified in the venom. The species codes in green, blue and black belong to *Africonus*, *Varioconus* and *Kalloconus*, respectively.

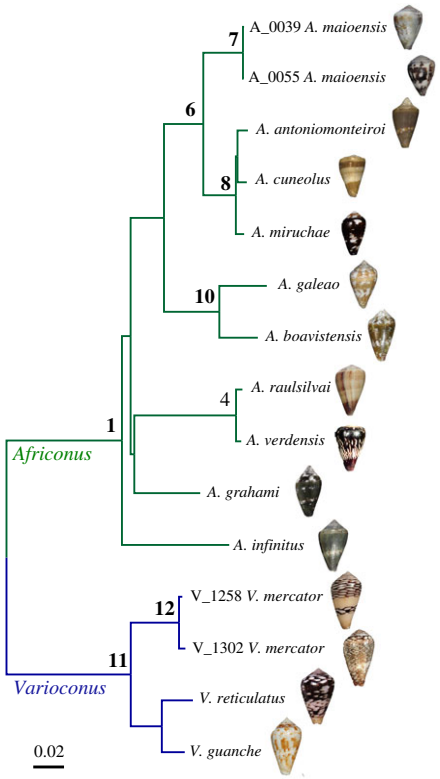
in the venoms of West African cones several members of the cysteine-rich secretory, antigen 5 and pathogenesis-related 1 (CAP) protein family. These proteins are often secreted and have protease activity with extracellular endocrine or paracrine function in a wide range of animals including venomous ones such as ants, wasps and snakes [42]. These proteins were previously found in the molluscivorous *Cylinder textile* and *Conus marmoreus*, and may be important for venom function ([43,44]; electronic supplementary material, figure S3).

(b) Variations in venom composition according to phylogenetic divergence

The venom compositions of the 15 specimens of *Africonus* and *Varioconus* were pairwise compared at different taxonomic levels (electronic supplementary material, figure S4). The two specimens of *A. maioensis* (A_0039 and A_0055) and those of *V. mercator* (V_1258 and V_1302) shared 51 and 53 conotoxin precursors, respectively, which represented 24–32% of the total sequences (electronic supplementary material, figure S4). The shared sequences between pairs of species from the same lineage within *Africonus* (see tree in electronic supplementary material, figure S1) were 2–35, with a mean of 11.9 (7% of the mean total sequences); between pairs of species from different lineages within the same genus (either *Africonus* or *Varioconus*) were 1–39 with a mean of 8.5 (5% of the mean total sequences); and between genera were 11 (0.7–1.9% of the total sequences (electronic supplementary material, figure S4).

An ancestral character state reconstruction analysis was performed to infer most likely ($p > 0.95$) conotoxin precursors at the different common ancestors (internal nodes) in the phylogeny using *K. trochulus* (K_0010) as outgroup (electronic supplementary material, table S3) and to detect potential instances of convergence (see full list of conotoxins found in more than one sample in electronic supplementary material, table S4). Inferred shared conotoxin precursors were preferentially concentrated in the nodes at the tips of the phylogenetic tree (figure 2). Many corresponded to the common ancestors of the pairs of individuals of *A. maioensis* (A_0039 and A_0055) and *V. mercator* (V_1258 and V_1302), respectively. In addition, an important number of shared-derived conotoxin precursors was inferred at the ancestors of (i) the closely related species *A. verdensis* (A_0239) from Santiago and *A. raulsilvai* (A_0031) from Maio; (ii) *A. galeao* (A_0048) and *A. boavistensis* (A_0520) from Boa Vista and (iii) the three species from Sal (*A. antoniomonteiroi* (A_0885), *A. cuneolus* (A_0855) and *A. miruchae* (A_0875); figure 2). Two precursors (Cerm_03 and Cerm_10) were inferred to be present at the ancestor of clade IV of *Africonus*. One (Pmag_02) and 26 (most prominently M, O1, O2 and T) conotoxin precursors were inferred to be present at the common ancestors of *Africonus* and *Varioconus*, respectively (figure 2). According to the inferred phylogeny, there could be several potential cases of convergence, such as transcript 646 (O2), present in *A. maioensis* (A_0055) and in *V. mercator* (V_1302); transcript 368 (O1), found in *A. maioensis* (A_0039) and in *V. guanche* (V_CG13); or transcript 2124 (O1), shared by *A. boavistensis* (A_0520) and *K. trochulus* (K_0010; electronic supplementary material, table S3). Likewise, within *Africonus*, the common presence of transcript 221 (T) in the common ancestor of *A. raulsilvai* (A_0031) plus *A. verdensis* (A_0239) and the distantly related species *A. maioensis* (A_0039 and A_0055) could be due to convergence.

According to the MCA, species from each genus clustered together in the two-dimensional scatter plot for the presence/absence of conotoxin superfamilies (figure 3). The species *V. reticulatus* (V_1278) appeared on the extreme lower end of axis 1, but it was not statistically considered an outlier. *Varioconus* species had negative Axis 1 scores, whereas *Africonus* species had positive or very slightly negative scores. The PCA of the relative abundance (percentage of the number of members) of each superfamily revealed no overlapping between genera (figure 3). According to the loadings of PC1 and PC2, *Varioconus* species had more abundant M, T and O1 superfamilies, whereas in *Africonus* the pattern was more disperse, with O2, P, Cerm_03 and A superfamilies as major



node 1	node 4	node 6	node 7	node 8	node 10	node 11	node 12
151 Pmag02	44 O2	336 Cerm10	48 O2	152 Pmag02	96 T	1562 Cerm10	802 Conkunitzin
	141 H	495 Cerm03	100 Conkunitzin	549 O2	127 Tpra06	1565 M	1559 Unassigned 04
	152 Pmag02		157 M	1065 Conkunitzin	521 T	1567 O2	1570 I4
	168 Cerm03		167 Conkunitzin	1076 V	524 T	1568 O2	1574 Conkunitzin
	188 Cerm10		169 O2	1077 V	525 T	1569 A2	1585 T
	213 O1		185 C	1089 Cerm03	529 Cerm02	1576 Con-ikot-ikot	1598 O1
	221 T		221 T	1112 M	535 O2	1591 O1	1618 M
	230 O2		228 O2	1115 B1	552 Cerm11	1599 T	1622 O3
	254 O2		267 M	1116 U	554 Cerm03	1600 T	1630 O1
	256 O2		299 Conkunitzin	1132 O2	556 J	1608 M	1632 O1
	257 B2		345 O2	1148 Rimp01	558 U	1638 O1	1636 Cerm10
	260 O1		356 L	1154 M	566 Y	1643 T	1637 O1
	263 O1		363 O2	1163 O1	570 M	1660 O2	1642 T
	270 M		364 Cerm03	1185 Conkunitzin	579 B2	1674 O3	1644 T
	272 F		365 O1	1199 T	583 O2	1697 Unassigned 04	1655 Rmil01
	276 O2		366 O1	1204 Cerm11	587 V	1703 Cerm03	1657 D
	285 Cerm03		371 O1	1216 O2	595 I1	1707 Cerm03	1659 O2
	299 Conkunitzin		373 A	1225 T	608 Con-ikot-ikot	1708 C	1666 M
	305 Conkunitzin		376 I1	1227 O1	609 Con-ikot-ikot	1712 Pmag02	1667 O2
	325 Unassigned 07		386 O1	1240 Q	611 O1	1715 O2	1669 O2
	327 O3		397 O1	1256 Cerm10	613 O1	1716 Conkunitzin	1671 A
	338 Conantokin F		399 O1	1287 A2	619 P	1723 O1	1672 Conkunitzin
	341 M		403 O1	1296 Cerm04	622 Conkunitzin	1766 O1	1673 H
			409 M		629 Rimp01	1778 E	1678 Rimp04
			411 M		631 Pmag02	1785 M	1685 I1
			416 T		635 Conkunitzin	1811 M	1687 J
			422 Cerm04				1688 H
			423 Cerm04				1690 Tand01
			424 Cerm04				1692 Cerm01
			425 O2				1704 Cerm02
			433 Unassigned 01				1714 S
			442 U				1718 I2
			443 U				1719 P
			444 U				1722 Rimp03
			445 Conkunitzin				
			456 M				
			465 F				
			467 Conantokin F				
			469 Conantokin F				
			473 Rimp02				
			476 E				
			478 Unassigned 04				
			481 O2				
			482 K				
			488 I2				
			491 J				
			493 Cerm03				
			499 Cerm10				

Figure 2. Maximum-likelihood ancestral reconstruction of conotoxin precursors along the phylogeny of cones from West Africa (see electronic supplementary material, figure S1). Statistical supports for inferred shared-derived conotoxin precursor are provided in electronic supplementary material, table S3. The table on the right shows the conotoxin precursors that are shared derived at a particular node (see electronic supplementary material, table S4). The red numbers are common conotoxin precursors found in distantly related nodes, which could represent potential instances of convergence. (Online version in colour.)

contributors (electronic supplementary material, figure S5). A discriminant function analysis (DFA) using PC1 to PC3 as variables and genus as factor, classified correctly 100% of the cases (also in the jackknifed classification test). Finally, relative expression levels showed no significant differences between both genera, although *V. reticulatus* (V_1278) and *V. mercator* (V_1258) were considered outliers, with overexpression of the T superfamily, as indicated by the strong positive loadings along PC1 (figure 3).

(c) Differential conotoxin expression patterns

A total of 11 superfamilies were detected as differentially expressed between *Africonus* and *Varioconus*: A2, B1, Cerm_02, Cerm_11, N, Rmil_02, S and V superfamilies were overexpressed in *Africonus*, whereas Cerm_01, K, and T were in *Varioconus*. After a Kruskal–Wallis test, only the overexpression of B1, Cerm_01, Cerm_11 and V superfamilies in *Africonus* remained significant ($p < 0.05$; electronic supplementary material, table S5).

The same tests found 29 superfamilies differentially expressed between vermivorous (*Africonus* and *Varioconus*) and piscivorous (*Chelyconus* and *Pionoconus*) genera (figure 4*a*). All but four were confirmed by the Kruskal–Wallis test (figure 4*a*; electronic supplementary material, table S5). Among those confirmed, the A ($p = 0.02$) and S ($p = 0$) superfamilies were overexpressed in both *Chelyconus* and *Pionoconus*, and the A2 ($p = 0.04$) in *Pionoconus*. The I5 superfamily was overexpressed in *P. magus* ($p = 0.005$), but this superfamily has only been reported in this species (figure 4*a*).

PCA on the relative abundance of conotoxin superfamily members in the venom clearly separated piscivorous and vermivorous genera (figure 4*b*). The former showed negative loadings along PC1, with most important contributions from M and Conkunitzin superfamilies, followed by S, A and B1. For the vermivorous genera, Cerm_03 and O2 superfamilies were the most important contributors to the positive loadings (electronic supplementary material, figure S6). DFA using PC1 to PC3 and diet as factor classified correctly 100% of the cases (also in the jackknifed classification test). Additionally, within the piscivorous cones, the PCA separated *Pionoconus* (Indo-Pacific Ocean) from *Chelyconus* (Atlantic Ocean). The former had M and Conkunitzin superfamilies as main contributors to the negative loadings whereas O2 and T superfamilies contributed to the separation of *Chelyconus* (not shown).

4. Discussion

(a) Conotoxin precursor assembly and annotation

The use of Illumina short reads to sequence cone venom gland transcriptomes has boosted the identification of conotoxin precursors [13]. However, *de novo* assembly is not straightforward and the use of various approaches may render strikingly different results. This should be taken into account when reporting the full venom catalogue of a species. In this regard, long read sequencing (paradoxically the now discontinued 454 but not PacBio or ONT technologies, which had not been yet applied to venom gland transcriptomes), which capture full-length peptides, and proteomic approaches have confirmed the exceptional variability of conotoxin precursors and complexity of venom cocktails. Another delicate step is annotation, which entirely relies on the quality of the reference database [45]. On one hand, the actual number of conotoxin

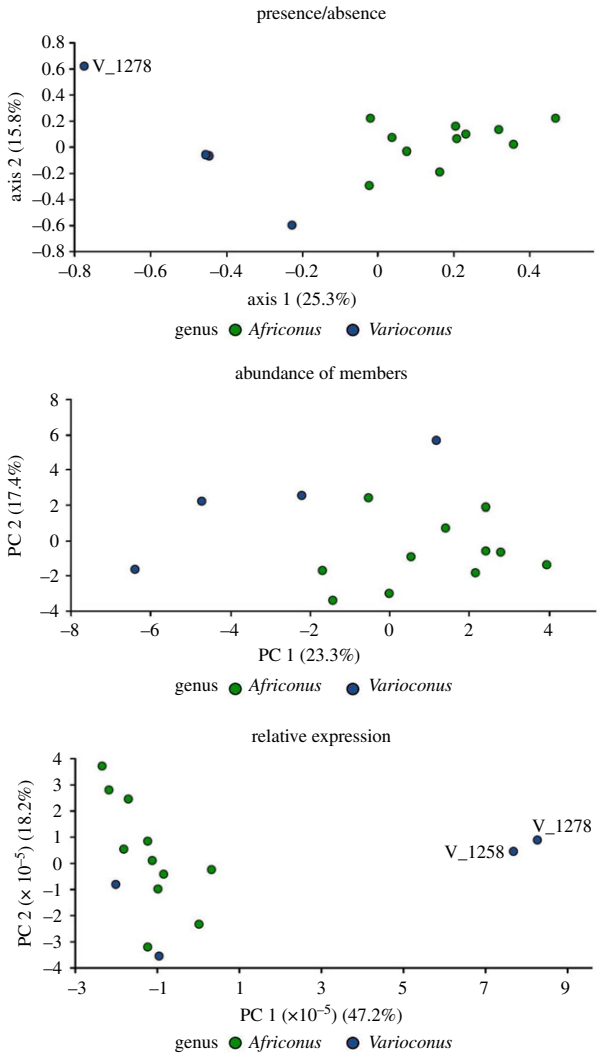


Figure 3. MCA and PCA comparing venom compositions of genera *Africonus* and *Varioconus*. The two-dimensional scatter plots are shown. The venom composition was defined as presence/absence of conotoxin precursor superfamilies (*a*); superfamily conotoxin precursor abundance expressed as percentage over total number of members (*b*) and relative expression (TPM) levels (*c*). The percentages of the eigenvalue (MCA) or variance (PCA) for each of the axis are indicated on the corresponding labels. Extremes and outliers are labelled. (Online version in colour.)

precursors could be underestimated. Low-expressed conotoxin precursor transcripts may not be recognized in the absence of counterparts in the reference database, if these were not detected in proteomic analyses, which are less sensitive [13]. On the other hand, we focused here on the possibility that assembly artefacts could overestimate conotoxin diversity [8,19,29]. This is particularly worrisome as, if not detected, these annotation errors could dangerously propagate once incorporated into updated reference databases [7,8,45]. We carefully inspected all the ORFs rendered by the BLASTX searches. At the assembly level, we often found regions of the assembled transcript (particularly at the 5′- and 3′-ends) mapped only by few reads that could lead to frame shifts and generate spurious variability [19]. At the annotation level, we implemented a TBLASTX step, which found two main sources of conotoxin misidentification (electronic supplementary material, file S2): (i) translations into the wrong frame, as exemplified by the R superfamily, originally described in *Conus marmoreus* [41], which once translated into the correct frame corresponds to the proteasome subunit alpha; and (ii) chimeric transcripts generated during the assembly. This is the case of several conotoxin precursors identified in *Darioconus episcopatus* [13]. For example, one precursor (BAS24857; named Cerm_18 in [8]) had the typical mature domain associated with T superfamily whereas the putative signal and pro-peptide domains, once translated into the correct frame corresponded to a sodium- and chloride-dependent glycine/GABA transporter. The here identified annotation errors (electronic supplementary material, file S2) should be eliminated from future reference databases.

(b) Venom composition and evolution

The analysed venoms contained 108-221 conotoxin precursors, which is in good agreement with numbers reported for other species of cones [6–8,25,29]. Comparison of venom repertoires revealed a larger set of expanded superfamilies in *Africonus* than in *Varioconus*. Although, it has been proposed that larger sets of conotoxins are associated with broader diets [19,22], we could not test this hypothesis, as the breadth of the worm diet of the different *Africonus* and *Varioconus* species is largely unstudied. Ecological studies on *Miliariconus miliaris* showed that the individuals of this species inhabiting the remote Eastern Island presented a considerably broader diet of worms, which could have evolved through ecological release in the absence of congeners [46]. This hypothesis could apply to *A. verdensis* (A_0239), which had a large conotoxin catalogue with many expanded superfamilies, and lives alone in Santiago Island.

Conotoxins are well known for their accelerated rates of evolution, which in turn generate high-sequence divergences even between individuals of the same species [6,8,25]. This is the basis of the reported general lack of common peptides between cone species, and the extended notion that virtually each species produces a unique venom cocktail [7]. The present study brings, for the first time, the opportunity to test the taxonomic limits of this hypothesis by comparing closely related species sharing relatively recent common ancestors. Individuals of the same species showed around one-fourth common conotoxin precursor sequences. This proportion is similar to those reported for intraspecific comparisons in *Dendroconus betulinus* [6], *Rhombiconus imperialis* [25] and *C. ermineus* [8]. The proportion of shared sequences decreased substantially for the pairwise comparisons between closely related species, within the range of 2–9%, in agreement with that reported for sister species of the genus *Turriconus* [7]. At the genus level, only 0.7–1.9% of the total sequences were common. Altogether, our results support that a phylogenetic signal remains in venom composition above the species level, but it is quickly eroded as lineages diverge and no identical conotoxin precursors are generally shared between closely related genera [19]. However, it is striking that several identical conotoxin precursor sequences were found between species from distantly related genera within Conidae (electronic supplementary material, file S1), indicating that those sequences are either subjected to strong balancing selection or reflect cases of convergent evolution. The rather erratic distribution of some of these sequences in the phylogeny of Conidae favours the latter hypothesis.

The two genera showed very contrasting results in the ancestral reconstruction analyses: only Pmag_02 could be traced back to the ancestor of *Africonus* whereas members of 14 conotoxin precursor superfamilies were inferred for the common ancestor of *Varioconus*. This discrepancy could be

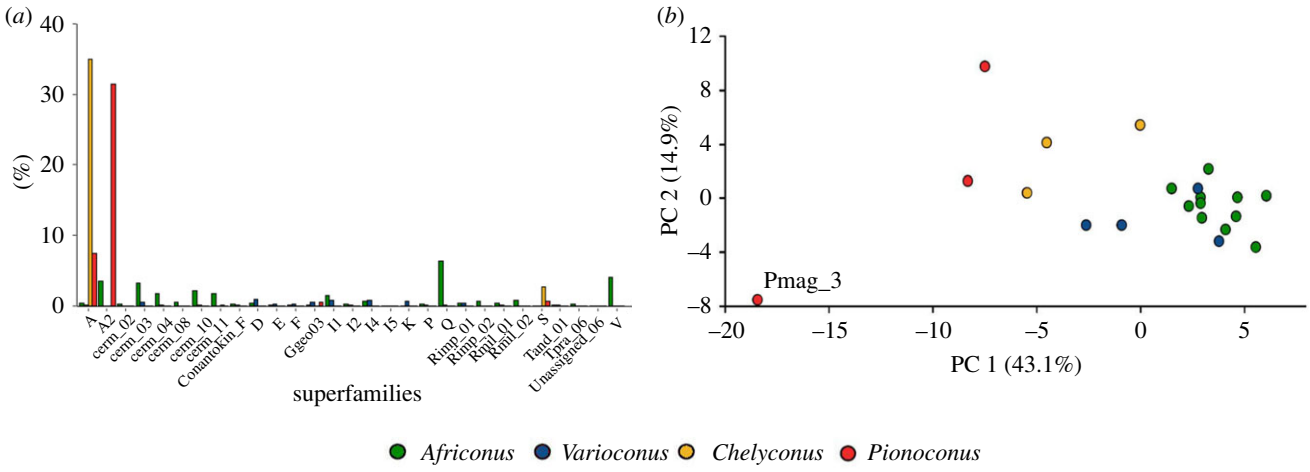


Figure 4. Differences in venom compositions of vermivorous and piscivorous cones. (*a*) Average expression (measured in TPMs) of conotoxin precursor superfamilies per genus. Vermivorous genera (*Africonus* and *Varioconus*) were compared to piscivorous genera (*Chelyconus* and *Pionoconus*). The bar plot depicts those superfamilies differentially expressed between diets. (*b*) PCA comparing conotoxin precursor abundance per superfamily between vermivorous and piscivorous species. The percentages of variance for each of the axis are indicated on the corresponding labels. In both panels, the genera *Africonus*, *Varioconus*, *Chelyconus* and *Pionoconus* are depicted in green, blue, yellow and red, respectively.

due to the larger number of taxa analysed and the greater diversity of members within the expanded superfamilies in *Africonus*. In any case, M, O1, O2 and T superfamilies were characterized by having five or more members in all studied species. The wider presence of these superfamilies in any cone and always showing similar levels in diversity of members [6–8,19,41] may suggest that the ancestor of living cones already had this core set, and that having members of these superfamilies (not necessarily the same) is essential either for defense (if, as proposed, this was the ancestral role of cone venom; [12]) or for triggering the minimum physiological responses necessary for the capture of a prey, regardless of whether it is a worm, a snail or a fish.

PCA and MCA have been used in several other animal groups to summarize the information related to venom composition [14,15] but not in cones to the best of our knowledge. MCA of presence/absence of superfamilies and PCA of the relative abundance of superfamily members recovered non-overlapping patterns for *Africonus* and *Varioconus*, indicating that species that are more closely related tend to have the same conotoxin precursor superfamilies and in similar proportions. By contrast, the PCA for expression levels did not find differences between the two genera, which may indicate functional convergence at this level, in agreement with the common expression patterns of conotoxins found in closely related Indo-Pacific vermivorous cone species that could not be explained by phylogeny but by functional convergence [47].

(c) Differential expression levels of conotoxins between genera and diets

Although the exact worm species eaten by the different species of *Varioconus* and *Africonus* are unknown, at least the three clades described within genus *Varioconus* correlate with different morphologies of the radular teeth suggesting subtle diet specializations [27]. We tested whether the two genera showed differential expression of their venom components, which could be correlated with diet adaptations. The B1, Cerm_01, Cerm_11 and V superfamilies presented significantly different expression between *Africonus* and *Varioconus* after the Kruskal–Wallis test was applied. The B1 superfamily (Conantokin) was originally described in the piscivorous *G. geographus* and reported to provoke a 'sleeping' phenotype in vertebrates, but its function in vermivorous species has not been characterized [4]. The V superfamily was first identified in the venom of the vermivorous *Virgiconus virgo*, but there is no information regarding its function [4]. The Cerm superfamilies were recently described in *C. ermineus* [8] and their function remains unknown.

Similarly, we tested for differential expression between piscivorous and vermivorous cones. We found four superfamilies differentially overexpressed in the two piscivorous species (A, A2, I5 and S) after the Kruskal–Wallis test. Thus, these superfamilies may be essential for piscivory in cones. The importance of having different members of the A superfamily for hunting fish has been highlighted previously for several cone species, as well as instances of functional convergence between Indo-Pacific and Atlantic piscivorous cones [8]. The S superfamily was first identified in *G. geographus* and found to inhibit neurotransmitter receptors [4]. Later, it was reported as minor component of different cone species, not all necessarily hunting on fish. The A2 superfamily has been described very recently [7], and its pharmacological function remains unknown. Despite sharing the same cysteine pattern to the I4 superfamily of *C. ermineus*, the I5 superfamily was defined as new in *P. magus* because it had a distinct signal region [29]. The functions of both superfamilies are unknown.

The possibility of comparing venom catalogues of closely related species of cones within a phylogenetic framework paves the way to understand how the venom repertoires were assembled and evolve, as well as to discern the relative role of diet and defense as selective forces. Here, we focused on two well-known species radiations of West African cones. For the first time, not only did we established levels of divergence of venom compositions at different taxonomic levels (between individuals, species, main lineages, genera) but also detected shared conotoxin precursors and inferred their potential presence at most recent common ancestors. This allowed (i) disentangling orthologous from paralogous conotoxin precursors; (ii) identifying functionally convergent conotoxin

precursors; and (iii) distinguishing shared derived from plesiomorphic conotoxin precursors. Our results demonstrate that different genera (*Africonus*, *Varioconus*, *Chelyconus* and *Pionoconus*) show distinct venom toolkits in terms of type and member abundance of conotoxin precursor superfamilies but that these differences are less evident when expression levels are analysed. Diet might be the strongest selective factor determining the relative expression of each venom component, as suggested by the differential expression analyses, although the contribution of differential conotoxin expression to defense needs to be further understood.

Data accessibility. The transcriptome sequences were deposited at the SRA database of NCBI (https://www.ncbi.nlm.nih.gov/sra) under accession numbers SRR11807492-SRR11807507, Bioproject PRJNA631880. The nucleotide sequences of all venom proteins here identified are available in fasta format (electronic supplementary material, file S3). Sequences of all conotoxin precursors and other venom proteins are also available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.3n5tb2rdm [48].

Authors' contributions. R.Z. conceived the study; M.J.T., C.M.L.A. and R.Z. obtained the samples; S.A. generated, assembled and annotated the transcriptomes; S.A., M.J.T. and R.Z. analysed the sequence data. All authors participated in the writing of the manuscript.

Competing interests. The authors declare no competing interests.

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