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Cite this article: Fedosov A, Zaharias P, Puillandre N. 2021 A phylogeny-aware approach reveals unexpected venom components in divergent lineages of cone snails. *Proc. R. Soc. B* **288**: 20211017. https://doi.org/10.1098/rspb.2021.1017

Received: 29 April 2021
Accepted: 11 June 2021

Subject Category:
Genetics and genomics

Subject Areas:
bioinformatics, evolution, genomics

Keywords:
conotoxins, venom evolution, phylogeny, Conidae, *Conasprella*, *Pygmaeconus*

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5479639.

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A phylogeny-aware approach reveals unexpected venom components in divergent lineages of cone snails

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Marine gastropods of the genus *Conus* are renowned for their remarkable diversity and deadly venoms. While *Conus* venoms are increasingly well studied for their biomedical applications, we know surprisingly little about venom composition in other lineages of Conidae. We performed comprehensive venom transcriptomic profiling for *Conasprella coriolisi* and *Pygmaeconus traillii*, first time for both respective genera. We complemented reference-based transcriptome annotation by a *de novo* toxin prediction guided by phylogeny, which involved transcriptomic data on two additional 'divergent' cone snail lineages, *Profundiconus*, and *Californiconus*. We identified toxin clusters (SSCs) shared among all or some of the four analysed genera based on the identity of the signal region—a molecular tag present in toxins. In total, 116 and 98 putative toxins represent 29 and 28 toxin gene superfamilies in *Conasprella* and *Pygmaeconus*, respectively; about quarter of these only found by semi-manual annotation of the SSCs. Two rare gene superfamilies, originally identified from fish-hunting cone snails, were detected outside *Conus* rather unexpectedly, so we further investigated their distribution across Conidae radiation. We demonstrate that both these, in fact, are ubiquitous in Conidae, sometimes with extremely high expression. Our findings demonstrate how a phylogeny-aware approach circumvents methodological caveats of similarity-based transcriptome annotation.

1. Introduction

Marine gastropods of the genus *Conus* are renowned for their remarkable diversity [1,2] and complex hunting strategies enabled by elaborated and deadly venoms. *Conus* venoms comprise highly diversified neuro peptides (conotoxins), hormones and small molecules in species-specific combinations that are suited to the biology of the prey and associated with particular hunting strategies [3–7]. Currently, *Conus* venoms are being studied at an ever-increasing rate because of their potential to be developed as drug leads. This capitalizes on their ability to modulate or disrupt the functioning of ion channels and receptors in the nervous system of prey or potential predators, including vertebrates [8,9]. However, *Conus* venoms are equally interesting from an evolutionary biology prospective. Generally, each toxin constitutes an adaptive trait that possesses a single function, and can be easily quantified and remapped onto the genome [10,11]. These properties, magnified by the impressive species diversity in *Conus*, and by the documented complexity of venom in each species [1], make *Conus* venoms an ideal model for studying drivers and dynamics of molecular evolution.

Eight genera (herein referred to as *divergent Conidae*) are currently included in the family Conidae, in addition to *Conus* [12]. Among them, recent phylogenetic studies on the family [13,14] demonstrate that the genera *Conasprella*, *Californiconus*, *Pygmaeconus* and *Lilliconus* form a separate lineage, sister to *Conus*, whereas

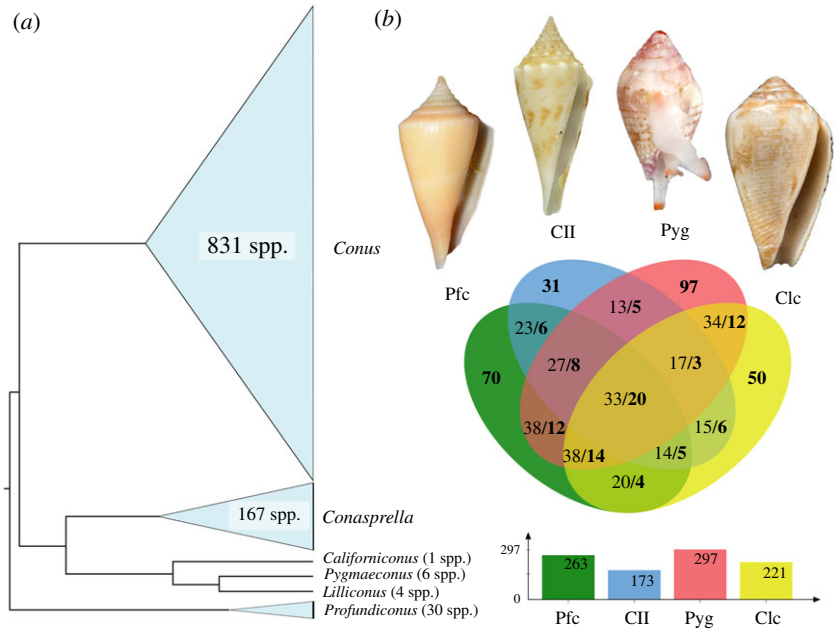

Figure 1. (*a*) Phylogenetic tree of the family Conidae (after Phuong *et al*. [14]). (*b*) Venn diagram showing numbers of SSCs shared by divergent Conidae genera. Shells of sequenced specimens (*Profundiconus*, *Pygmaeconus*), or conspecific to sequenced ones (*Conasprella*, *Californiconus*), shown above (not to scale). In the Venn diagram intersections: regular font—total cluster count; in bold—number of clusters with at least one transcript of moderately high or high expression (tpm > 100). (Online version in colour.)

Profundiconus is found to be the earliest diverging lineage of the family (figure 1*a*). Current knowledge of venom composition among these taxa is highly skewed [15]. While a wealth of data is available for *Conus* species and for the single species of *Californiconus*, *C. californicus* [5,16], only fragmentary data has been published for *Conasprella* [17,18], the first and only analysis of *Profundiconus* venom was published only recently [19], and virtually no data exist for other divergent Conidae. To fill this gap, we carried out the first comprehensive transcriptomic analyses for *Conasprella* and *Pygmaeconus* and we present the results here. Our results will foster analyses of the apparition and diversification of the venom component across the Conidae radiation. This is an important milestone on the way to understanding cone snail venom evolution.

Venoms of divergent Conidae have remained poorly studied for a reason. Unlike *Conus*, these genera are much less speciose, most of their species are rare and either dwell in deep water (most *Conasprella*, *Profundiconus*), or have very restricted distribution (*Lilliconus*), or are very small (*Pygmaeconus* and *Lilliconus*). These factors complicate sampling, and recovery of even one live specimen suitable for venom profiling is generally a stroke of luck, and it was the case with *Conasprella coriolisi* (Moolenbeek & Richard, 1995) and *Pygmaeconus traillii* (Adams, 1855) analysed here. This limited sampling posed a challenge to corroborating sets of predicted venom transcripts. Divergent lineages are expected to possess divergent venom components when compared to *Conus* venoms. Consequently, they are more difficult to identify by conventional approaches in peptide annotation based on sequence similarity and structural features [20,21], and increasingly rely on the *de novo* annotation. With only one specimen per species available, the accuracy of the *de novo* toxin prediction cannot be cross-validated by data from independently sequenced conspecific specimens, and predicted venom components also cannot be verified by means of proteogenomics [20]. So, to provide more robustness to the de novo transcript annotation, we tested a phylogeny-aware approach. This approach first helped to identify the divergent lineages in which one would expect to find divergent venom components. Next, the phylogeny was used to identify related taxa among which the clusters of transcripts predicted as venom components can be cross-validated. Finally, a phylogenetic approach was also legitimate in tackling the diversity of venom peptides, where the sequence of the signal region can serve as a proxy for precursor classification into gene superfamilies [22,23]. In essence, the same principles make up the theoretical framework of concerted toxin discovery [24,25], which, however, has never been convincingly performed within one study. Here, we show that by applying this approach, a large fraction of the venom transcript diversity overlooked by reference-based annotation can be identified. Furthermore, we demonstrate that some of these novel clusters are also present, and may be quite diversified in *Conus*. Yet they have been barely noted thus far because of the methodological caveats of the similarity-based transcriptome annotation. Finally, we discuss the impact of such previously undetected venom components on hypotheses related to the evolution of the cone snails and their toxins.

2. Material and methods

(a) Specimen collection

The specimen MNHN-IM-2013-47769, *P. traillii*, hereafter *Pygmaeconus*, was sampled in shallow waters off New Ireland during the KAVIENG 2014 expedition (expeditions.mnhn.fr). It was photographed and dissected alive. The venom gland was immediately suspended in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA), stored overnight at room temperature, and subsequently at −20°C. The specimen MNHN-IM-2013-66001, *C. coriolisi*, hereafter *Conasprella*, was collected by dredging at depths of 270–275 m during the KANACONO expedition (doi:10.17600/16003900; expeditions.mnhn.fr) off New Caledonia, west of the Isle of Pines. It was kept in chilled seawater, and

dissected alive upon arrival to the onshore laboratory. The venom gland was also preserved in RNAlater and stored at −20°C.

(b) RNA extraction and sequencing

RNA was extracted from venom glands of *Pygmaeconus* and *Conasprella* using the TRIzol reagent (Thermo Fisher Scientific) following the protocol provided by the manufacturer. Bioanalyzer traces were used to assess total RNA quality and determine suitability for sequencing. The cDNA libraries were prepared and sequenced either at the New York Genome Center (*Conasprella*) or at the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley (*Pygmaeconus*). In New York, libraries were prepared using the automated polyA RNAseq library prep protocol and sequenced with Illumina HiSeq 4000 with 150-bp paired-end reads, resulting in the acquisition of 15 029 852 150-bp paired-end reads. In Berkeley, the KAPA Stranded mRNA-Seq kit was used to synthesize cDNA, ligate adapters using TruSeq HT adapters and barcode samples, and sequenced on the Illumina HiSeq 4000 system, resulting in the acquisition of 30 063 937 100-bp paired-end reads.

(c) Transcriptome assembly

Adaptor removal and quality trimming of the *Conasprella* and *Pygmaeconus* raw reads were performed using Trimmomatic v. 0.36 [26] with the following parameters: ILLUMINACLIP option enabled, seed mismatch threshold = 2, palindrome clip threshold = 40, simple clip threshold of 15; SLIDING WINDOW option enabled, window size = 4, quality threshold = 15; MINLEN = 36; LEADING = 3; TRAILING = 3. The reads were then assembled using Trinity v. 2.11.0 [27] with the kmer size set to 31, which performs best to assemble venom gland transcriptomes of *Conus* [28,29]. The assembly metrics were checked using the TrinityStats.pl module. The same parameters were used to trim and assemble raw read data on *Profundiconus neocaledonicus* [19], hereafter *Profundiconus*, and *C. californicus* [5], hereafter *Californiconus* (electronic supplementary material, table S1). To quantify the abundance of the predicted transcripts we used the function rsem-calculate-expression [30], with bowtie2 [31] mapper to map the trimmed reads on the assembly. Transcripts-per-kilobase-million (tpm) values were used, as they are recognized as the most appropriate metrics of expression levels [5,23].

(d) Identification of putative conotoxin precursors

We applied three approaches to identify potential toxin transcripts in the assembled transcriptomes. First, we conducted a direct BLASTx search of the *Conasprella* and *Pygmaeconus* assemblies against an in-house toxin database. This database was obtained by combining all entries with the keyword 'toxin' from UniProt with all entries from ConoServer [32], and supplemented by lists of putative gastropod toxins of the tonnoidean *Charonia tritonis* [33], buccinoideans *Cumia reticulata* [34] and *Hemifusus tuba* [35], non-conid conoideans *Clavus canalicularis* and *C. davidgilmouri* [36], and cone snails *Profundiconus* spp. [19], *Conus ermineus* [23], *Conus magus* [37], *Conus tribblei* [38], *Conus praecellens* [29], *Conus betulinus* [39] and *Conus litteratus* [40] plus the 15 species of *Conus* and *Californiconus*, analysed by Phuong *et al*. [5]. Then alignments of the contigs that produced reliable hits (BLAST PID > 0.55, with aligned length no less than half of the best matching database entry, and with no stop codons) were parsed from the XML output by the Python script1 and checked visually. After removal of flanking regions, these predicted transcripts were combined in dataset 1, comprising toxins with high sequence identity to known animal toxins, primarily, conotoxins.

Subsequently, we performed coding DNA sequence (CDS) prediction, using ORFfinder [41]. Only CDSs comprising 35 or more amino acid (AA) residues, and starting with either 'ATG' or alternative initiation codons were output. We ran SignalP v. 5.0 [42] to identify a subset of CDSs with signal region prediction (*D*-value > 0.7), and further filtered this subset to remove CDSs with a transmembrane domain (identified by Phobius v. 1.01 [43]). Then we removed redundant CDSs derived from predicted alternative isoforms of the same transcript: CDSs showing less than two AA-residue divergence were removed to keep only the CDS corresponding to the most highly expressed isoform (in-house Python script2). The resulting catalogue of CDSs was used for structure-based search via HMMER v. 3.2.1 [44] against the Pfam database [45]. The CDSs with HMMER hits were then sorted, based on the relevance of the HMMER annotations to the venom functions. These annotated CDSs made up dataset 2. In general, three broad classes were recognized: toxins (t), hormones (h) and enzymes and other peptides with known or proposed function in envenomation (p).

Datasets 1 and 2 contained sequences with detectable sequence identity and/or with structural similarity to known venom peptides. But, we expected from the divergence between *Conus* and the analysed divergent Conidae that highly divergent clusters of venom components were lacking from these datasets. As we had only one specimen per species analysed, we employed a phylogenetic approach to *de novo* toxin identification. The scope of the search was defined to include all lineages of Conidae outside the genus *Conus*, i.e. the genera *Profundiconus*, *Conasprella*, *Californiconus* and *Pygmaeconus* (figure 1*a*). First, the non-identical CDSs, containing a signal sequence, but no transmembrane domains were recovered from the reassembled datasets of *C. californicus* and *P. neocaledonicus*, following the same methodology, as for *Conasprella* and *Pygmaeconus*. The trimmed reads were then remapped on these CDSs and coverage-per-base was calculated, as a measure of the reliability of predicted CDSs. Those CDSs with the smallest per-base coverage value below 3 were removed from the dataset. The signal sequences of the thus filtered CDSs (numbering in total 16 906) were pooled into a single file and clustered using CD-Hit v. 4.8.1 [46]. The signal sequence is the most conserved region of a conotoxin precursor and is widely used for conotoxin classification [47], and phylogenetic clustering of gene superfamilies [22]. Therefore, we considered the recovered signal sequence-based clusters (hereafter, SSC) as potential gene (super)families of secreted peptides. The three alternative identity thresholds of 0.6, 0.65 and 0.7 used for clustering correspond to the range of signal sequence PID in most canonical gene superfamilies based on ConoServer [47]. The alternative clustering schemes were evaluated based on the already annotated transcripts from the dataset 1, to make sure that transcripts representing distinct toxin gene superfamilies, on no occasion end up in a single SSC. The set of 13 616 SSCs obtained with the identity threshold of 0.65 was found to best separate known gene superfamilies, so it was selected for the subsequent analyses. All the SSCs containing three or more transcripts, represented in at least two genera of divergent Conidae, and showing moderately high (100 < tpm < 1000) or high (tpm > 1000) expression levels in at least one genus were identified (in-house Python script3). These SSCs were aligned separately using MAFFT v. 7.475 [48] with G-INS-1 strategy and 'unalignlevel' parameter set to 0.2. The cleavage sites were predicted by ConoPrec [47], and the Cys-patterns were identified by in-house Python script4. When screening such clusters, the following conditions were checked: (i) predicted signal sequence lacking long repeats of one or two residues, such as 'LLLLLLLLL', 'LSLSLSLSLSLS' or 'VSVSVSVSVSVSV', (ii) complete precursor not exceeding 200 AA, (iii) mature region comprising over 20 AA, (iv) consistent alignment features within each SSC. Some identified clusters might, however, correspond to transcripts of house-keeping genes, including transcripts translated into the wrong frame [37]. To filter these out, we used BLASTx (*E*-value 10–5) to search the nucleotide sequences of the SSCs against the

SwissProt manually curated database [49]. The clusters that did not return a match were aligned to the best-match ConoServer entry using the built-in AA search tool, and either ascribed to known venom peptide gene superfamilies, or designated as novel gene superfamilies. The transcripts identified by the analysis of SSCs and lacking from datasets 1 and 2 formed dataset 3. The final lists of venom peptides were compiled for *Conasprella* and *Pygmaeconus* by combining datasets 1, 2 and 3.

(e) Analysis of the distribution of novel gene superfamilies in species of *Conus*

To determine whether the novel gene superfamilies identified from the SSCs in divergent Conidae are also present in *Conus* species, we first reassembled 15 *Conus* transcriptomes (electronic supplementary material, table S1), and remapped trimmed reads to the resulting assemblies, using the same methodology as for the divergent Conidae datasets. We then ran CAP3 (with default parameters) followed by CD-Hit (PID 99%) to reduce assembly redundancy. The clustered assemblies were used in BLASTx against the database of novel gene superfamilies from dataset 3. To roughly estimate the contribution of the novel gene superfamilies to the toxin expression in each *Conus* species, one known highly expressed conotoxin gene superfamily was selected for each species to serve as a reference (electronic supplementary material, table S1). All available sequences of this gene superfamily (specifically including sequences identified in the original study) were added to the new gene superfamily database. The BLAST results (*E*-value of −10) were first sorted by in-house Python script5 in the following manner: (i) the query transcripts were assigned to the reference superfamily if the PID exceeded 85% while the aligned length constituted no less than 0.7 of the length of the best matching entry from the BLAST database, and tpm expression level exceeded 5; (ii) the query transcripts were provisionally assigned to a novel gene superfamily if PID exceeded 30%, while the aligned length was no less than 0.6 of the length of the best matching sequence in the database, and the tpm exceeded 5. Transcripts that fulfiled these conditions were aligned by gene superfamily, and then each alignment was screened to remove erroneously assigned transcripts. Then expression levels were summed up for each gene superfamily, and relative expression (in per cent) calculated. Based on these data we performed a principal component analysis (PCA) to evaluate the degree of venom composition similarities among the analysed Conidae. The PCA diagram was constructed with PAST v. 4.06 [50], using the variance–covariance method.

3. Results

(a) Venom composition in *Conasprella* and *Pygmaeconus*

Direct similarity search with BLASTx identified 96 *Conasprella* transcripts with high similarity (BLAST PID above 55%) to known *Conus* venom components included in the in-house toxin database. Among these, 80 were counterparts of *Conus* venom peptides referable to 17 gene superfamilies (electronic supplementary material, table S2). The search of predicted CDS against the Pfam-A HMM domain database revealed a diversity of additional transcripts with proposed functions in venom. Most numerous among them were transcripts bearing Von Willebrand factor domains, and various peptidases (M, C, S), both with typically low expression levels (tpm < 40). A total of 81 predicted *Pygmaeconus* venom transcripts were identified by BLASTx. Of these only 44 were counterparts of the *Conus* venom peptides and represented 15 gene superfamilies. Among other revealed components, most notable were diversified transcripts with high similarity to neuropeptides: APWGamide, cerebrin, elevenin, FFamide, FMRFamide, FxRIamide, LASGLVamide-4, LFRFamide-2, NdWFamide-2, Wwamide, all with low expression (tpm < 20), except NdWFamide-2 (tpm 120)—electronic supplementary material, table S2. HMMER analysis predicted a diversity of additional peptides with previously suggested functions in venom: astacin, peptidases C, M, S and trypsin-like, chitinase, CAP, ShK and peptides containing Von Willebrand-like domains.

The 13 616 SSCs were filtered to select only those comprising three or more predicted transcripts, found in at least two species of divergent conid genera (figure 1*b*), and highly expressed in at least one genus. The 90 SSCs that fulfiled these criteria were manually curated to exclude clusters comprising transcripts that did not show features of toxins, leading to a final set of 71 SSCs. Of these, 37 clusters contained CDSs identified by direct BLASTx from *Conasprella* and/or *Pygmaeconus*, and already assigned to known gene superfamilies. Another 22 SSCs comprised transcripts assigned to known toxin gene superfamilies but missed by BLASTx. These clusters added 25 (23.6%) and 19 (29.7%) new transcripts of known gene superfamilies to the catalogues of *Conasprella* and *Pygmaeconus*, respectively. Among them, seven and five known conotoxin gene superfamilies in *Conasprella* and *Pygmaeconus*, respectively, were only identified from these SSCs, and were thus lacking in datasets 1 and 2. Seven more SSCs are diversified in the divergent Conidae genera, are highly expressed in at least one studied transcriptome, and demonstrate canonical conotoxin precursor structure [15], but do not show similarity to any established conotoxin superfamily. These are designated as new conotoxin superfamilies DivCon 1–7. The transcripts of known and of newly designated gene superfamilies of conotoxins constitute the datasets 1 and 3. In *Conasprella*, datasets 1 and 3 contain 74 and 24 transcripts, respectively, whereas in *Pygmaeconus*—40 and 47 transcripts, respectively.

The final catalogues comprise 170 and 190 venom components classified in 29 and 28 gene superfamilies in *Conasprella* and *Pygmaeconus*, respectively (electronic supplementary material, table S2). Of them, 116 and 98 transcripts from *Conasprella* and *Pygmaeconus*, respectively, represent known or new conopeptide gene superfamilies, classified into four groups: (i) 'canonical' gene superfamilies and common classes of *Conus* venom peptides, such as conkunitzin, conodipine, conophysin, conoporin, (ii) 'divergent' gene superfamilies, largely known from *C. californicus* [5,16], plus the recently identified very taxonomically restricted gene superfamilies New-Geo-1 [51], and Pmag02 [37] (iii) novel gene superfamilies, sharing structural properties of conotoxins, (iv) putative conotoxins (figure 2). The latter group comprises unrelated transcripts with structural similarity to known conotoxins detected by HMMER, which are not assigned to any gene superfamily. A total of 86 predicted *Conasprella* transcripts were assigned to 16 'canonical' gene superfamilies, with dominating P- (18 transcripts), M- (11), O2- (9) and I2- (8) gene superfamilies (figure 2*a*). A total of 19 transcripts are identified in six 'divergent' gene superfamilies accounting for 20.3% of the summed toxin expression, and seven predicted transcripts in four novel gene superfamilies account for only 2.45% of the summed toxin expression. Notably, fewer transcripts of the 'canonical' gene superfamilies (47) are identified in *Pygmaeconus*, and almost half of them (21 transcripts) represent the T- superfamily,

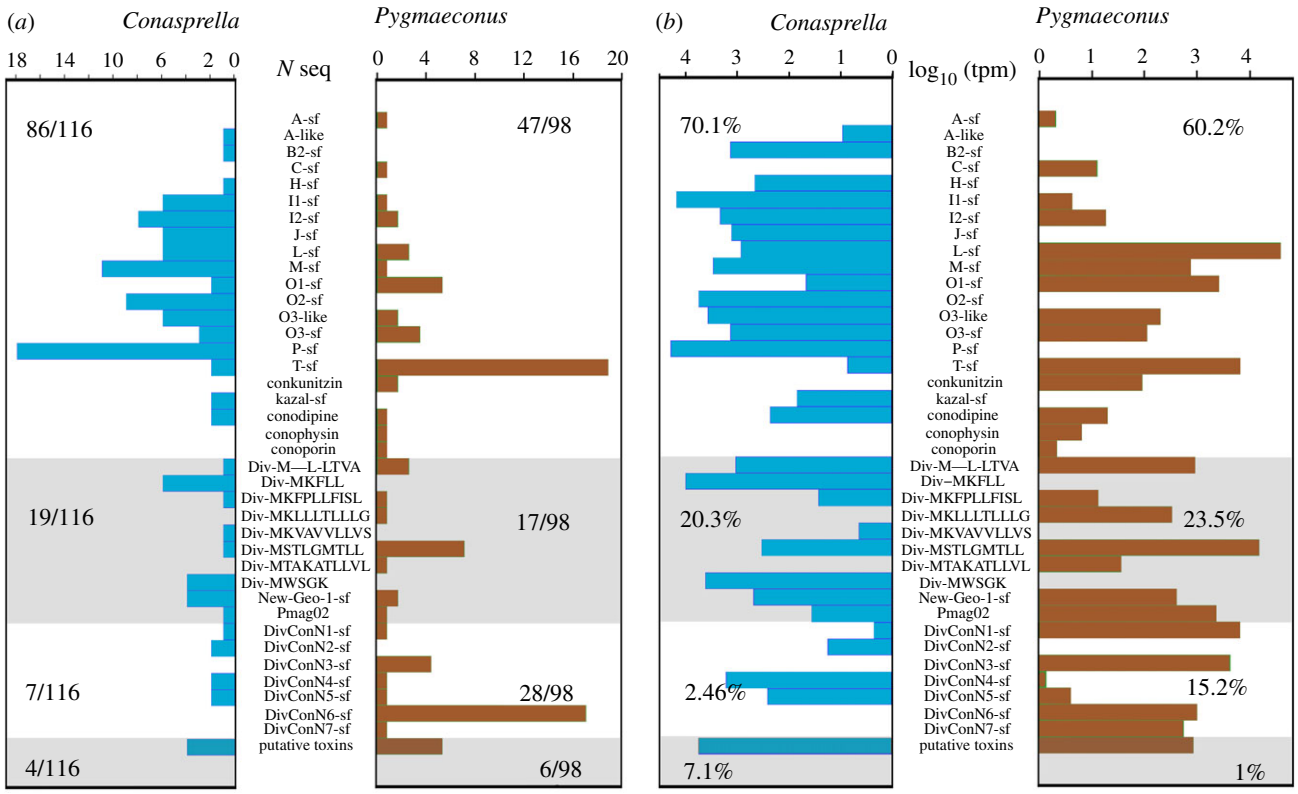

Figure 2. (*a*) Counts of the identified transcripts by gene superfamily for *Conasprella coriolisi* (left) and *Pygmaeconus traillii* (right). (*b*) Log_{10} transformed relative expression levels of the conotoxin gene superfamilies in *Conasprella coriolisi* (left) and *Pygmaeconus traillii* (right). (Online version in colour.)

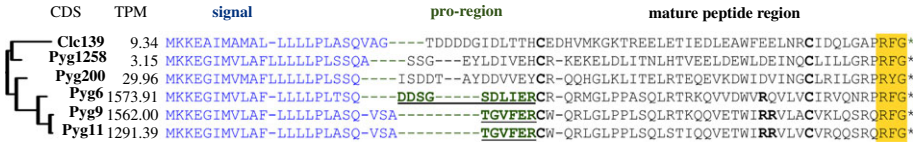

Figure 3. Alignment of the DivConN3 gene superfamily. The neighbour-joining phylogenetic tree (obtained with MEGA v. 6 [52]) on the left based on the complete precursor sequences. Signal sequences shown in blue and pro-region underlined green; bold letters correspond to Cys residues and predicted cleavage sites within mature peptide region; shading marks C-terminal RF-amide motif. (Online version in colour.)

followed by O1- (6), L- (3) and O3- (3). Among the seven 'divergent' gene superfamilies identified in *Pygmaeconus*, the Divergent-MSTLGMTLL (eight transcripts) is the most diversified and is by far the most highly expressed (18.6% of the summed toxin expression). Novel gene superfamilies in *Pygmaeconus* are represented by 28 predicted transcripts, that contribute 15.2% to the summed toxin expression.

(b) Novel gene superfamilies identified through clustering of the signal region

Seven SSCs, comprising in total 55 putative toxins of divergent Conidae are designated as novel gene superfamilies (electronic supplementary material, figures S1–S7 and table S3). All the predicted transcripts of DivCon2 are cysteine-free, and DivCon3, DivCon5 and DivCon7 show conserved arrangement of Cys residues. The remaining three gene superfamilies vary in the arrangement of Cys residues in the mature peptide region, and members of DivCon1 and DivCon4 also display highly divergent pre- and mature peptide regions. However, variations in the length and Cys pattern are also found in many 'canonical' conotoxin gene superfamilies, (i.e. A-, I2-, M-, O1-), and is reflected in diversified functions of the included gene families (see e.g. [37]).

Among the novel toxin gene superfamilies, ConDiv3 is notable for the peculiar sequence of its six members, bearing an Arg-Phe-Gly motif (RF-amide) C-terminally. ConDiv3 is only detected in the transcriptomes of the *Californiconus* and *Pygmaeconus* clade (figure 1*a*), and in the former is represented by a single low expression transcript (tpm 9.34). Of five transcripts identified in *Pygmaeconus*, three show high expression, with tpm values exceeding 1000–1500. These three precursors Pyg6, Pyg9 and Pyg11 form a distinct cluster (figure 3) and are distinctive in that they possess a pre-region (underlined in figure 3) and have an internal cleavage site within the predicted mature peptide region.

(c) Diversity and expression of novel gene superfamilies in *Conus*

We hypothesized that members of the novel gene superfamilies DivCon 1–7 may also be present in *Conus*, but overlooked, since their published transcriptome annotations mainly relied on similarity-based search (BLAST). Furthermore, the discovery of the gene superfamilies New-Geo-1

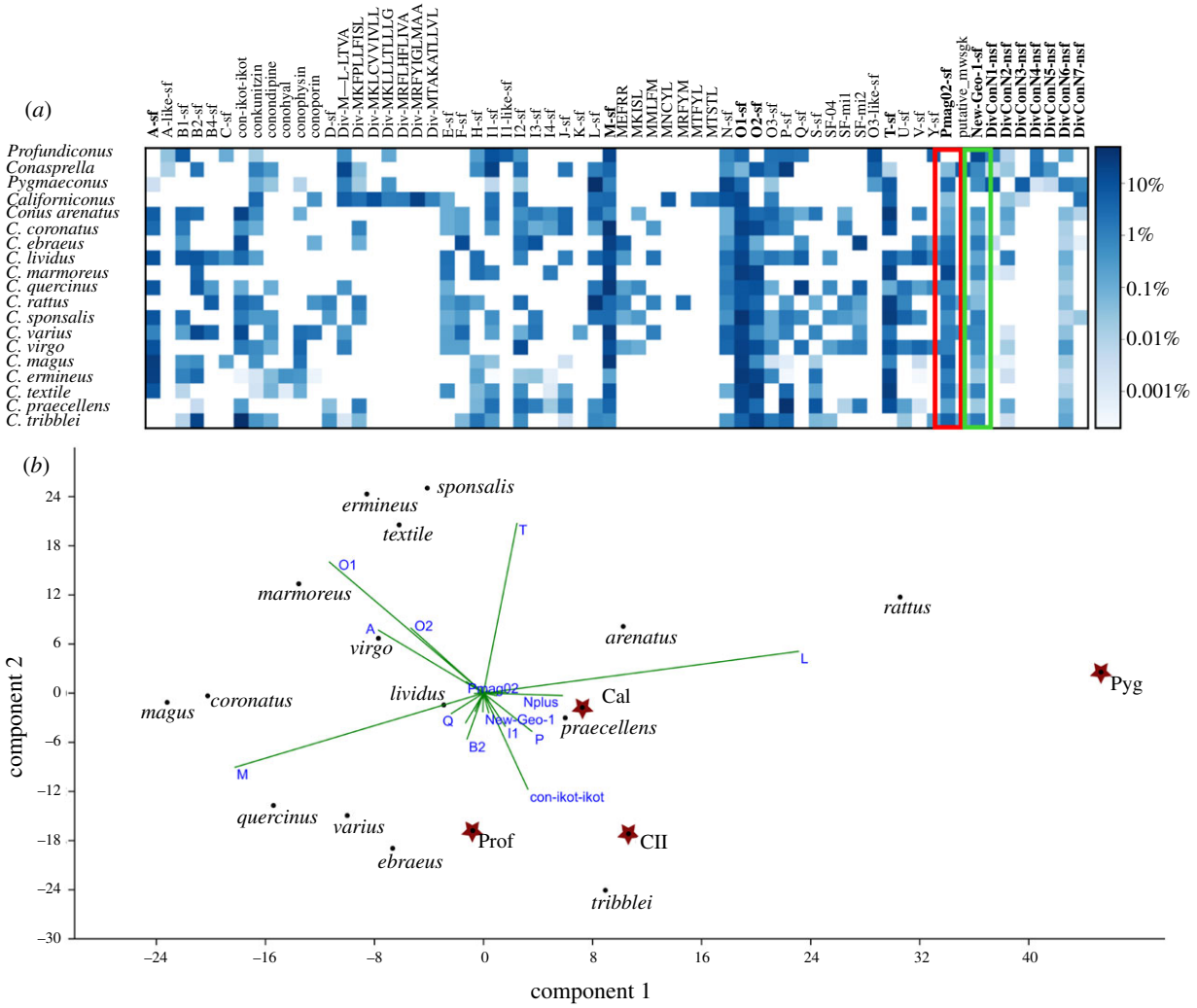

Figure 4. (*a*) Heat map of the gene superfamily expression in the four species of the divergent Conidae and 15 species of *Conus*. Pmag02 and New-Geo-1 are highlighted. (*b*) PCA diagram of the divergent Conidae (stars Prof - *Profundiconus*, CII - *Conasprella*, Cal - *Californiconus*, Pyg - *Pygmaeconus*) and 15 species of *Conus* (dots) based on gene superfamily expression data (electronic supplementary material, table S4). Principal components 1 and 2 account for a total of 42.27% of the observed variation. The position of *Pygmaeconus* at the extreme of PC1 is mainly due to the contribution of L- and next N-/Divergent-MSTLGMTLL-gene superfamilies. The placement of *Conasprella* is largely explained by the contributions of con-ikot-ikot- and M-gene superfamilies. (Online version in colour.)

and Pmag02 in divergent Conidae requires corroboration. We, therefore, evaluated the distribution of these gene superfamilies in 15 species of *Conus* representative of both its phylogenetic diversity (12 different subgenera) and the known dietary guilds (worm-, fish- and mollusk-hunters).

Counterparts of DivCon2 are identified in transcriptomes of six *Conus* species, with a single transcript in each species, usually with moderately high expression levels ($100 < \text{tpm} < 1000$). Additional DivCon2 members are revealed in what Li *et al*. [21] referred to as the 'putative MTKLL' gene superfamily in *Conus lenavati*, *C. caracteristicus* and *C. betulinus* (electronic supplementary material, figure S2). Similarly, incomplete precursors, but ones obviously closely related to those in the DivCon7 superfamily, are detected in *Conus arenatus* and *C. sponsalis*. The precursor Im20.1 of *C. imperialis* [53] is also clearly referable to the ConDiv7 superfamily (electronic supplementary material, figure S6).

The gene superfamilies New-Geo-1 and Pmag02 are present in all, and DivCon6 in almost all the analysed *Conus* species. Both the Pmag02 and the New-Geo-1 superfamilies are represented by multiple transcripts per species. Even after removal of the minor isoforms that are less than two AA residues divergent from the closest major isoform of the same species, final datasets of Pmag02 and the New-Geo-1 comprise 34 and 83 sequences, respectively. The New-Geo-1 superfamily reaches highest relative expression in *Conus ebraeus*, *C. sponsalis*, *C. textile* and *C. tribblei*, and contributes about 1% of the total toxin expression in each of these species (figure 4*a* and electronic supplementary material, table S4). Pmag02, in general shows even higher expression, contributing 1–3% to the summed expression in most *Conus* species, but with a maximum of notable 15% in *C. marmoreus*.

4. Discussion

The venoms in *Conasprella* and *Pygmaeconus* differ notably from each other in terms of dominant venom gene superfamilies: P- and O3- in *Conasprella*, versus T- and L- in *Pygmaeconus*. PCA analysis of the data in electronic supplementary material, table S4 suggests that the *Pygmaeconus* venom is highly divergent from other Conidae venoms (figure 4*b*). This can be explained by the phylogenetic distinctiveness of *Pygmaeconus*, but also by the small size of the animal compared to all other Conidae included in the analysis. *Pygmaeconus* venom evolution might have been driven by adaptation to an

uncommon niche among Conidae, and thus to a different spectrum of interactions from those in larger Conidae. Further studies on the feeding biology and diet of both *Conasprella* and *Pygmaeconus* are needed to corroborate this hypothesis.

The 98 and 58 transcripts of known gene superfamilies identified for *Conasprella* and *Pygmaeconus*, respectively, are well within the diversity range reported in the single-transcriptome studies on *Conus*. These numbers slightly exceed those reported for *P. neocaledonicus* (55—[19]), but a much higher diversity is reported for *Californiconus* (185—[5]). In part, this can be explained by biological factors, such as dietary breadth, varying among taxa, with the most diverse diet found in *Californiconus* [5]. Despite our efforts, we believe that the venom diversity reported for *Conasprella* and *Pygmaeconus* is, to some extent, an underestimate, resulting from the limited data available to us. Analyses based on a single transcriptome typically report fewer toxins, and discrepancies may sometimes be striking. For example, 53 toxins were identified from *C.* (*Pionoconus*) *consors* [54] as opposed to a total of 232 toxins in three separately sequenced specimens of the closely related *C.* (*Pionoconus*) *magus* [37]. Furthermore, in our *de novo* CDSs annotation, we prioritized the reliability of the toxin identification, and so we used rather stringent filtering criteria. The N-terminally incomplete CDSs were ignored, as well as those with low probability of signal region prediction (*D*-value < 0.7), low expression, as well as clusters with less than three predicted transcripts, or exclusive to one taxon. Nevertheless, in both *Conasprella* and *Pygmaeconus*, a quarter to almost one-third of the known gene superfamily members could only be identified from the annotation of predicted SSCs, but not from the search against reference databases. Most likely, this is partly a result of the high PID value we used to limit the output of the initial BLASTx step, and by relaxing it, we might have been able to identify more toxins at the first step of the annotation. However, when we tried relaxing the PID or BLASTx *E*-values, it led to huge outputs with increasing proportions of false positives, and their manual curation was not feasible. An array of algorithms, known as machine learning and recently developed into the automated pipeline *Conus*Pipe [21], offers yet another way to optimize toxin identification. This tool showed excellent performance when trained on *Conus* datasets and applied to the identification of *Conus* toxins. Nevertheless, we found it methodologically incorrect to use a training set of sequences derived from *Conus*, and then apply it to datasets of notably divergent taxa. Still, most of the parameters used by *Conus*Pipe, were either set explicitly, or checked at the stage of SSC screening. Despite this semi-manual procedure allowing us to improve recovery of toxin sequences, additional transcriptomic and proteomic data, including on other species of divergent Conidae, will be important to corroborate our findings.

Of particular interest is the gene superfamily DivCon3 identified by the annotation of the SSCs, a likely innovation of the *Californiconus–Pygmaeconus* subclade of Conidae. Due to the cleavage sites within the mature region (monobasic in Pyg6 and dibasic in Pyg9 and Pyg11), we hypothesize that the final peptide products of these three precursors are 13–14 AA-long oligopeptides bearing a RF-amide motif C-terminally. Because of both the presence of a C-terminal RF-amide and the very small size, the predicted cleavage products of the ConDiv3 are similar to conorfamides [20,55,56]. A pronounced physiological effect was demonstrated for the conorfamide CNF-Vc1 from *C. victoriae*. In mice, it elicits increase of intracellular calcium levels in the dorsal root ganglia and causes nearly complete muscle paralysis [20]. A similar pharmacology may characterize DivCon3 members, and the high expression of these transcripts in the venom gland of *Pygmaeconus* implies their functional significance. Further functional studies on these oligopeptides are necessary to identify their molecular targets. By contrast, the signal region of ConDiv3 does not show any similarity to that of conorfamides, and mature peptide regions of ConDiv3 bear two conservatively arranged Cys residues, whereas known conorfamides are cysteine-free. This suggests that DivCon3 and conorfamides of *Conus* are likely convergently evolved venom components and constitute yet another remarkable parallelism in the molecular evolution of toxins in Conidae.

Despite the fact that research on the chemical structure and pharmacological properties of conopeptides commenced over four decades ago [57], the complexity of *Conus* venoms may still be greatly underestimated. Recent studies have demonstrated that defense-invoked venoms may differ in composition from predation-invoked venoms [3]. Likewise, some *Conus* feeding strategies involve the release of a subset of venom components directly into the water to alter the behaviour of their prey prior to injection of a killing shot of venom. The physiologically active components in this subset may be as exotic as specialized insulins, or small molecules mimicking the natural pheromones of the prey [7,58]. This suggests that there may be a great diversity of venom components, or specific enzymes involved in the biosynthesis of these components that are still not identified. The reason for this is largely methodological—most venom analyses use similarity-based searches, as they primarily target canonical conotoxin gene superfamilies, and (at best) peptides of similar structural properties.

Our phylogeny-aware approach on a subset of *Conus* species, in which specific divergent and/or taxonomically restricted venom components are sought out in different lineages of cone snails, revealed a previously uncharacterized diversity of putative toxins even in what might seem to be well-annotated transcriptomes. Remarkably, New-Geo-1 and Pmag02 appear to be ubiquitous in *Conus* as well as in the divergent Conidae genera. This case shows how an inaccurate picture of venom components distribution across the Conidae evolutionary tree can bias research hypotheses related to toxin evolution. The New-Geo-1 and Pmag02 superfamilies were previously known to be highly expressed in the fish-hunting subgenera *Gastridium* and *Pionoconus*, respectively, and so might be interpreted as specific adaptations to piscivory. If this were correct, the very high expression levels of New-Geo-1 and Pmag02 in non-piscivorous *Profundiconus* and *Pygmaeconus*, respectively, could suggest that these components are a part of the defensive venom targeting fish predators. However, recently, transcripts referable to Pmag02 were also identified in the vermivorous *Conus* lineages from West Africa [6]. Finally, as a phylogenetically more representative picture of New-Geo-1 and Pmag02 distribution in Conidae emerges with our study, any support evades for the hypothesis that these gene superfamilies are at all related to piscivory.

On the one hand, we emphasize a need for thorough and accurate annotation of transcriptomic data, even if it requires laborious tasks that cannot be fully automated. On the other hand, we must admit that further studies that are solely based on—OMICs data are deemed to remain somewhat incremental, because they are unable to produce functional data.

Lacking such data, the roles of various venom components remain unclear, and with it the benefits that a particular taxon acquires by evolving them. Major breakthroughs in understanding drivers of Conidae venom evolution should thus be guided by a knowledge of the feeding ecology of different species of Conidae and require functional assays alongside venom profiling. A major challenge along the way is the further elaboration of existing methodologies to overcome common shortages of research samples and, increasingly, improving behaviour documentation practices to eventually analyse molecular data within an ecological context.

Data accessibility. The transcriptomic sequencing data are deposited in the NCBI SRA database, under the Bioproject PRJNA735765. Sequences of the predicted toxins are provided in electronic supplementary material, table S2 and figures S1–S8, Python scripts are available at https://github.com/Hyperdiverseproject/Divergent_Conidae. The data are provided in the electronic supplementary material [59].

Authors' contributions. A.F.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, writing—original draft, writing—review and editing; P.Z.: resources, writing—review and editing; N.P.: conceptualization, funding acquisition, resources, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

Funding. The present study was supported by the Russian Science Foundation, grant no. 19-74-10020 to A.F., and by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement no. 865101) to N.P.

Acknowledgements. The material was collected during the KANACONO expedition in New Caledonia (convention MNHN-Province Sud, APA_NCPS_2016_012; PI N. Puillandre and S. Samadi) and the KAVIENG 2014 expedition in Papua-New-Guinea (endorsed by the New Ireland Provincial Administration and operated under a Memorandum of Understanding with the University of Papua New Guinea; PI P. Bouchet and J. Kinch), as part of the Our Planet Reviewed and the Tropical Deep-Sea Benthos programmes, organized jointly by the *Muséum national d'Histoire naturelle* (MNHN), Pro-Natura International (PNI) and the *Institut de Recherche pour le Développement* (IRD). The organizers acknowledge funding from the Total Foundation, the *Laboratoire d'Excellence Diversités Biologiques et Culturelles* (LabEx BCDiv, ANR-10-LABX-0003-BCDiv), the *Programme Investissement d'Avenir* (ANR-11-IDEX-0004-02), the *Fonds Pacifique*, CNRS' *Institut Ecologie et Environnement* (INEE) and the project CONOTAX, funded by the French *Agence Nationale de la Recherche*—France (ANR-13-JSV7-0013-01). These expeditions operated under the regulations then in force in the countries in question and satisfy the conditions set by the Nagoya Protocol for access to genetic resources. We thank Laetitia Aznar-Cormano for her help with the RNA extraction of the *Pygmaeconus* sample. We are thankful to Giulia Fassio (University of Roma) for providing intermediate data files on *Profundiconus*, and to Helena Safavi-Hemami (University of Utah) for her comments on the novel gene superfamilies. We are thankful to Claudia Ratti for her comments on the manuscript.

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