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Article

Cone Snails Broad-Transcriptomics Elucidate the Evolutionary Diversification and Anti-Microbial Potential of Conopeptides

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Abstract: *Conus* venoms are both highly powerful and complex exhibiting a remarkably intriguing molecular variability. The biologic reasons behind such astonishing molecular diversity are yet to be fully understood. We hypothesized that the current knowledge has been hampered by a lack of studies targeting the whole *Conus* genus backed by a feeding habit analysis, as opposed to the abundant studies focused on single species or at the individual level. We aim to enlighten the understanding of the remarkable venom variability in cone snails while pushing to deliver novel peptides for biomedical applications through a broad transcriptomics approach. Here, we assessed 76 publicly available venom-related and unrelated transcriptomes from a total of 20 different *Conus* species. The shared transcriptomic repertoire discovered revealed several gene variations in accordance with predatory diets (e.g., gene loss in piscivorous species), indicating that feeding habit largely influences venom evolution. Furthermore, evidences of ubiquitous symbiotic relationships within the venom organs were depicted, as biological processes alien to *Conus* species (e.g., Sorocarp morphogenesis) were found in all analyzed transcriptomes. Moreover, 88 potential anti-microbial peptides were bioinformatically detected, including one showing similarity with the human ACE2 receptor. Our study highlights the importance of in-depth comparative transcriptomic analyses, fostering cross-field synergic assessments by relying on informatic, biologic and pharmacologic resources.

Keywords: *Conus*; Transcriptomes; Venom; Anti-microbial peptides; Feeding habit; Evolution; ACE2

1. Introduction

Animal natural venoms are being studied for more than a century, but a particular group emerges entrancingly in this research field [1–3]. The highly diverse *Conus* genus has more than 700 species living in tropical and subtropical regions worldwide [4–8]. As predators, they have three main diets, consisting of either fishes, molluscs or worms, which they catch with their neuroactive venom amidst rocky shores and coral reefs [9,10]. Their predatory venom contains about two hundred toxins known as conotoxins [11]. Synthesized as precursors, these toxins undergo a series of post-transcriptional modifications (PTMs) enabling them to interact specifically with their target, which includes the presynaptic membrane calcium channel, potassium and calcium channels, the receptors of serotonin and adrenal hormone, among others [11–18]. Nowadays, more than 8,000 conopeptides have been discovered out of 50,000 to 1 million estimated to exist [19,20]. For predation, generally compounds from A, M, O1 and T gene superfamilies account for the bulk of venom cocktails, but there is a surprisingly large variation not only between species but also between individuals

[1,2,15,21]. Additionally, venom profiles change throughout different venom discharges, as while peptides from the O-superfamily are prominent in most profiles, peptides of the M- and T-superfamilies are only prevalent in subsequent injections [1,2,6,15,22]. This variability is reflected in the conotoxin genes, as there is a discrepancy between their number and their transcripts. Perhaps due to natural variation coupled with PTMs, highly expressed transcripts are similar, but variation of less expressed sequences is also unusually large [1,2,21,23]. The main evolutionary drive behind the hyper inter and intra-specific variations remains unclear, but the conotoxin genes are proposed to develop by gene duplication events, followed by either positive selection resulting in the neofunctionalization of physiological genes in venom-related organs, or by a neutral force of sub-functionalization and restriction to venom-related organs [23–29]. Indeed, some venom-related proteins have much higher expression levels in the foot than in the venom gland, possibly showing the endogenous nature of these proteins [2]. There are also findings of toxin genes being expressed in venom-unrelated tissues in snakes and in platypus [24,30,31]. After duplication events, venom potency is enhanced via increased dosage and replenishment capabilities, inclusively allowing for greater target diversity [32,33]. Counter-intuitively, there are cases in which it is gene loss the main evolutionary trait resulting in specialization at the cost of reduced fitness [34,35]. Such is the case of rattlesnakes, whose loss of toxin genes constitutes the main force behind evolutionary changes in venom function [36].

In order to increase the comprehension of the variability of *Conus* venoms, we declined the previous overwhelming focus on single species and single individuals analyses and replace it with an innovative strategy consisting in gathering the greatest possible *Conus* dataset for searching similar aspects connecting the whole genus. Such aim could provide valuable insights on venom evolution, potentially helping unveil trends behind venom variability. A transcriptomic approach was chosen given its capability of indicating actively expressed genes as well as the functions of the proteins they encode at great speed and scale. These features are particularly informative for functional genomics research, especially in species like *Conus*, with still limited genetic resources available [37,38]. We conducted in-depth transcriptomic analyses (76 transcriptomes) with the aim of finding shared genetic signatures among the representatives of the 20 cone snail species studied. Informative patterns of gene variation (e.g., gene loss and reduced similarity) were revealed in accordance with predatory diet. This suggests that cone snail venom variability and evolutionary traits are better elucidated through the scope of a contextual feeding habit evaluation. Furthermore, the surprising discovery of shared transcripts unfamiliar to *Conus* genome supports the existence of extensive symbiose relationships [39–41]. Finally, we explored in silico the therapeutic potential of conopeptides by investigating their anti-microbial capabilities. Presently, several studies have been successful in inspecting various marine-derived peptides from venomous species including, for instance, cephalopods and cnidarians [42,43]. Here, our performed analysis relying on our vast library of annotations predicted 88 putative anti-microbial peptides (AMPs) in *Conus* species. Altogether, our study extended the current understanding of the *Conus* venom's evolution and functionalities.

2. Materials and Methods

This investigation made use of data acquired (from NCBI), polished (with Trimmomatic and Cutadapt), assembled (*de novo* generated using Trinity) and annotated (with GO Ontology) based on a previous work from our group [44]. The dataset consists of 76 transcriptomes from 20 different species (*Conus marmoreus*, *C. betulinus*, *C. consors*, *C. coronatus*, *C. ebraeus*, *C. ermineus*, *C. imperialis*, *C. judaeus*, *C. lenavati*, *C. literatus*, *C. lividus*, *C. magus*, *C. miliaris*, *C. quercinus*, *C. rattus*, *C. sponsalis*, *C. tribblei*, *C. varius*, *C. ventricosus*, and *C. virgo*): 69 are from venom duct and gland of 20 species, while the remaining 7 come from 5 other tissues (nervous ganglions, salivary glands, osphradium, foot and proboscis) of 2 species (*C. consors* and *C. ventricosus*).

In summary (Figure 1), the whole dataset was divided in two groups: one with transcriptomes from venom-related organs and another with transcriptomes from other body tissues. For annotation, the Swiss-Prot and Tox-Prot databases were used for peptide and toxin identification, respectively [38]. Afterwards, alignment and clustering resulted in creation of three phylogenetic trees (illustrating venom clusters). In the end, we evaluated the biomedical potential of conopeptides by targeting the venom-related and unrelated transcriptomes, as well as from sequences with 14 of the 29 annotations shared by all *Conus* species but exclusive to venom organs.

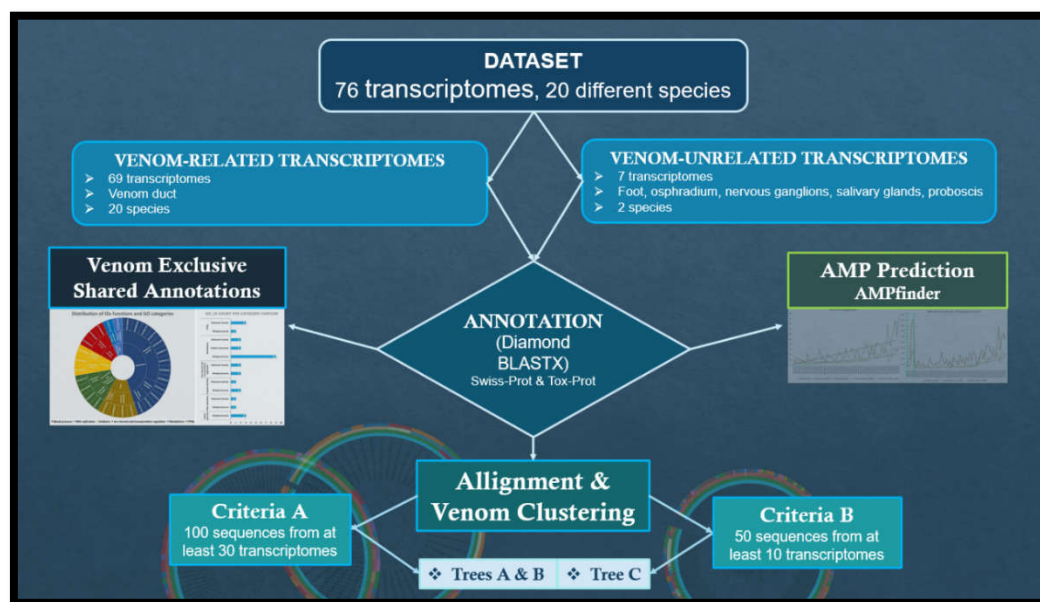


Figure 1. Methodology workflow.

The 69 venom-related and 7 venom-unrelated transcriptomes were annotated with Swiss-Prot and Tox-Prot (using Diamond BLASTX and MMseqs2, v15.6f452), for peptide and toxin identification, respectively [45,46]. From the list of 29 shared annotations, 14 were chosen due to higher venom-like specificity or possible symbiont origin (list in Appendix A).

Sequence alignment was built by Mafft (v7.490) with default parameters, then processed by UGENE [47,48]. Venom clustering processes were carried out with VSEARCH (v2.29.1 [49]) on the venom-related dataset according to two criteria, set as follows:

- Criteria A: 100 sequences from at least 30 transcriptomes; the selected sequences would be further annotated according to the database of Tox-Prot;
- Criteria B: 50 sequences from at least 10 transcriptomes; the selected sequences would be further annotated according to the database of UniProt-Trembl.

Next, phylogeny trees were elaborated with iTOL (<https://itol.embl.de/>) utilizing previous annotation lists with transcriptome number and its corresponding origin species, tissue, diet and blast target [50].

The biomedical prediction model utilized was AMPfinder, a bioinformatic tool which employed with its corresponding database is used to identify antimicrobial peptides (AMPs) [51]. This database is primarily based on experimentally verified AMPs collected from various public repositories, scientific literature, and computational analyses. To test the effectivity of a dedicated AMP finder and the advantages of using one, the first phase of AMP prediction targeted smaller the group of peptides with the chosen 14 annotations. Afterwards, anti-microbial potential was assessed in the whole transcriptomic dataset.

The R software was used to generate UpSet plots with the shared venom-related and unrelated annotations, which allow the visualization of all the intersections in a clean matrix layout [52]. In

addition, the R software was used to visualize the AMP distribution across the different feeding habits through a violin plot (Appendix B) and a Principal Component analysis (Appendix C). All data was normalized before being sorted according to the following “Min-Max” normalization formula:
$$\text{normalized data } (X') = \frac{X - X_{min}}{X_{max} - X_{min}}$$
. This formula helps reducing distribution issues, ensuring reliability in comparing variables across the different diets [53].

3. Results

3.1. General Transcriptomes Annotation and Biomedical Predictions

The annotation revealed a cumulative total of 553,878 peptides recognized by Swiss-Prot in the venom-related transcriptomes, with 5.2% of them (28,805) being toxin sequences recognized in the Tox-Prot database. In comparison, there were 108,466 cumulative peptide sequence annotations from transcriptomes coming from venom-unrelated tissues, with 1.75% of them (1,902) also being toxins (from the salivary glands’ transcriptome). In addition, a total of 88 potential AMPs were predicted, 15 unique to the venom-related transcriptomes (including 7 from the chosen 14 annotations), 17 unique to venom-unrelated transcriptomes and 56 shared between them. The Table 1 resumes the annotation results.

Table 1. AMP findings and transcriptome annotation summary including the cumulative number of peptides and toxins.

Transcriptomic group	Number of peptides	Number of Toxins	AMP predictions
Venom-related	553,878	28,805	71 (15 unique)
Venom-unrelated	108,466	1,902	73 (17 unique)

3.2. Shared Transcriptomic Repertoire

The 7 transcriptomes from nervous ganglions, salivary glands, osphradium, foot and proboscis share 5,913 annotations (GO IDs) in common, which are perceived to represent housekeeping genes (Figure 2 – A). Regarding the 69 venom-related files, they share 2,104 annotations (Figure 2 – B), most of which are also present in other tissues. The removal of the endogenous annotations from the group of shared annotations in the venom-related transcriptomes resulted in an assemblage of 29 shared but venom-exclusive annotations. They are related to six main aspects: DNA replication, metabolism, blood pressure, ion channel and transport regulation, PTMs and seemingly symbiotic beings (Figure 3). The latter is demonstrated by three peculiar annotations: GO:0031288, GO:1903665, and GO:1903669. In Figure 3 it is revealed that most of the annotations are related to a biological metabolism process, showing the backbone of conserved genomic expression is related to the logistical processes behind venom synthesis

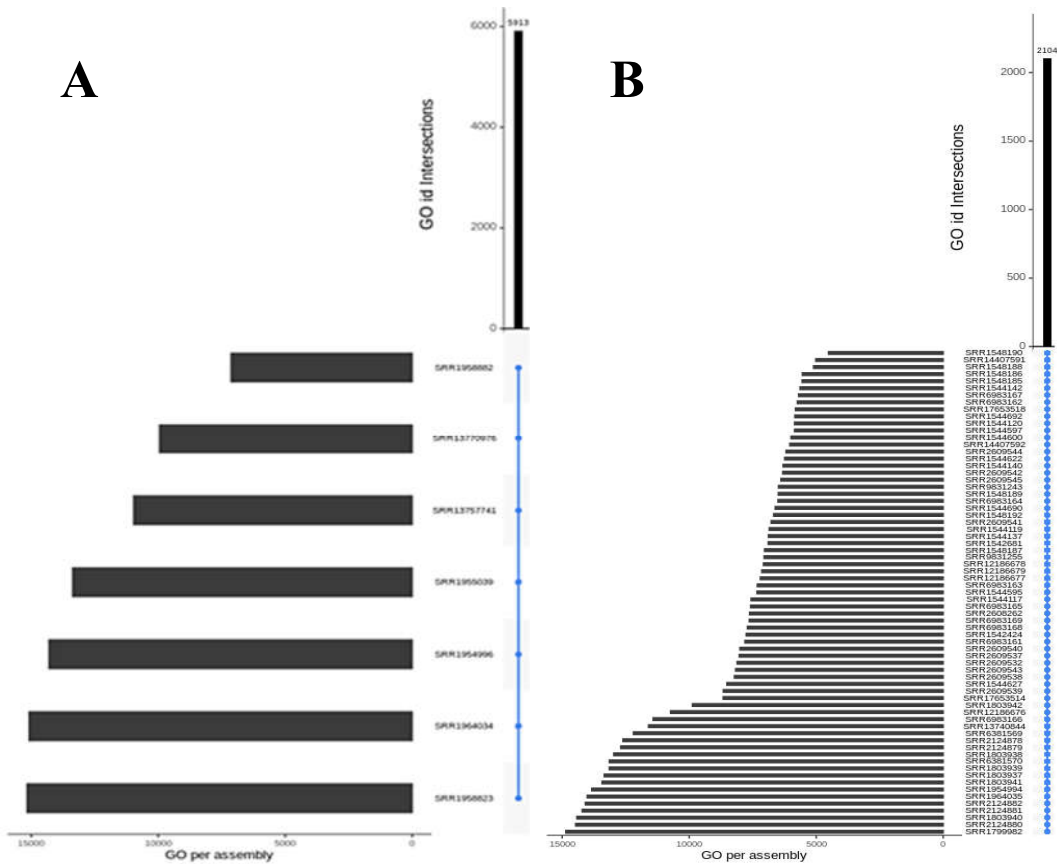


Figure 2. Shared annotations (GO IDs) in the venom-unrelated (A) and in the venom-related (B) transcriptomes. The blue vertical line intersecting all files indicates the shared nature of the annotations: 5,913 in A and 2,104 in B. The side black bars indicate the total annotations attributed to each transcriptome individually.

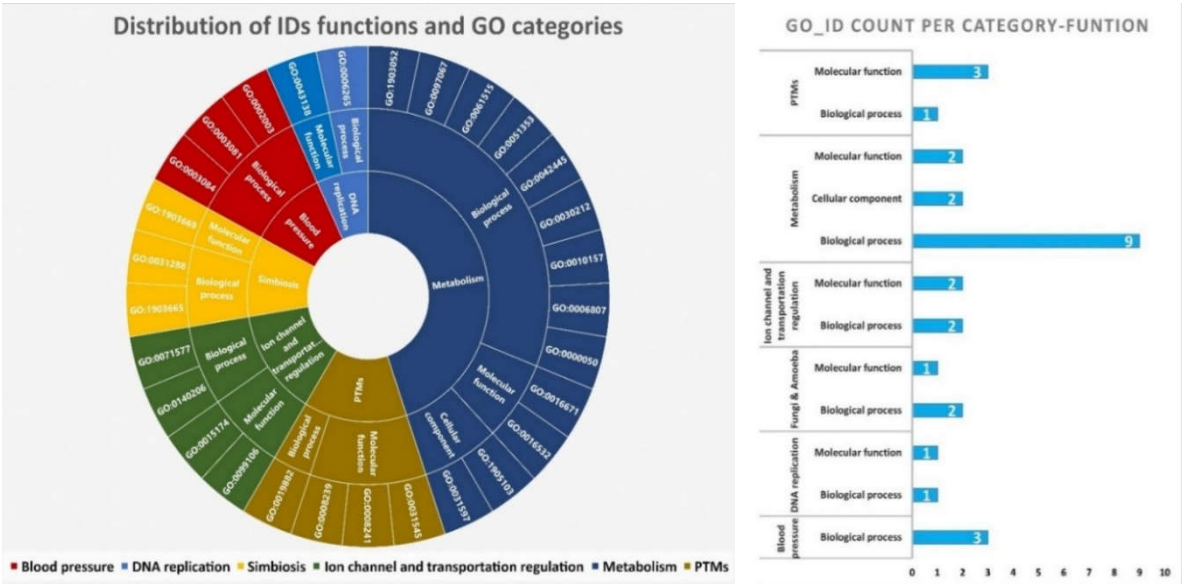


Figure 3. The left, circular graph shows the distribution of the 29 GO IDs according to their six main functions and their GO category (e.g., “Biological Process” or “Molecular Function”). The bar graph on the right indicates ID count by GO category and designated major function.

3.3. Phylogeny

Following criteria A, two Trees were created: Tree A with 271 branches (Figure 4) and Tree B with 117 branches (Figure 5). Both of these trees consist of venom duct sequences from a total of 12 species while the 21 different blast targets present an array of conotoxin peptides, as expected from the Tox-Prot database. All of these are conotoxins of the M-superfamily, which is the most structurally varied of the identified superfamilies and was detected in every species studied to date [54,55]. M-superfamily peptides are categorized into five branches, designated M1-5, based on the number of residues present in the third cystine loop situated between the fourth and fifth cysteine residues. These branches are further divided into two subgroups, on account of the number of residues in the mature peptide: Mini-M (1-3) and Maxi-M (4-5).

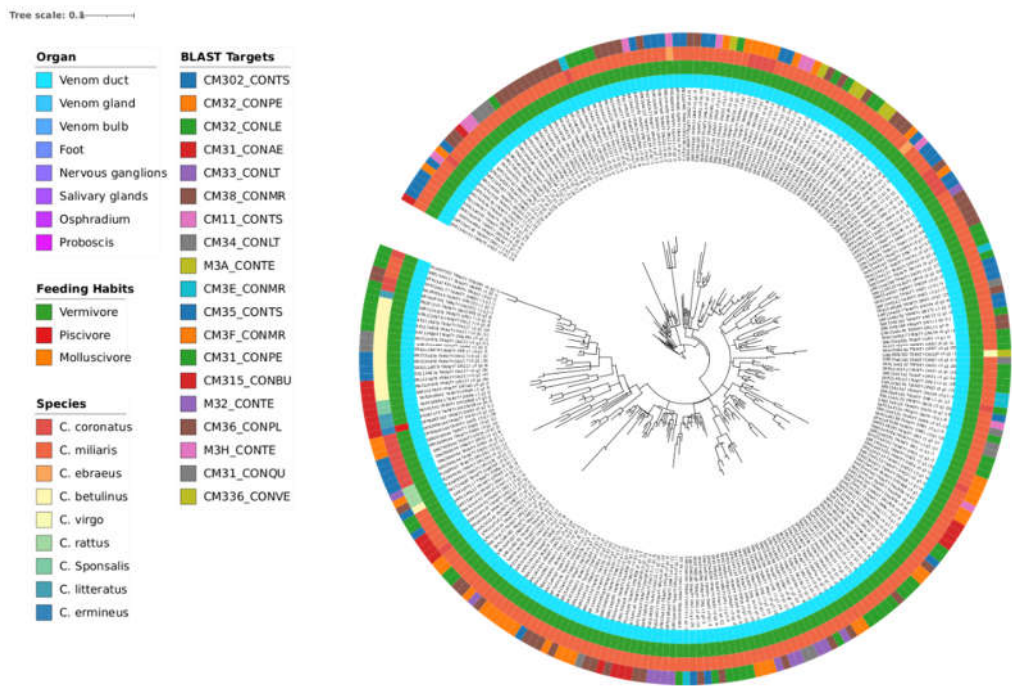


Figure 4. Phylogeny Tree A of 9 *Conus* species (*C. betulinus*, *C. coronatus*, *C. ebraeus*, *C. ermineus*, *C. litteratus*, *C. miliaris*, *C. rattus*, *C. sponsalis*, *C. virgo*) illustrating the clustering of 271 sequences (blasted with Tox-Prot database) from the vermivorous and piscivorous predatory diets. The annotation colour from the inner circle to the outer is the following: organ, diet, species and Blast target.

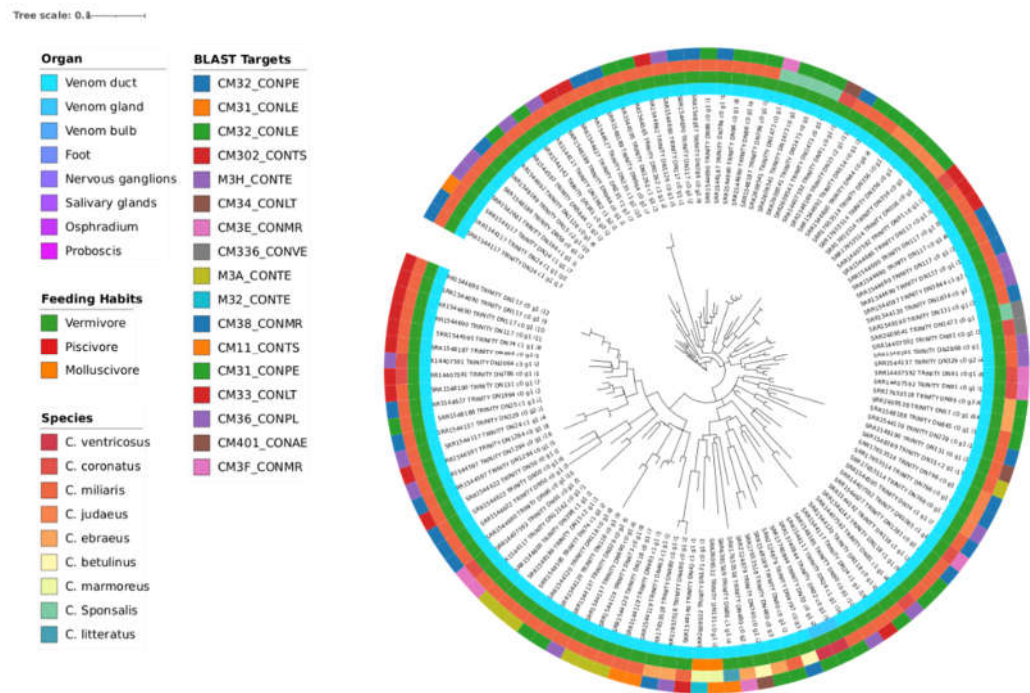


Figure 5. Phylogeny Tree B of 9 *Conus* species (*C. betulinus*, *C. coronatus*, *C. ebraeus*, *C. judaeus*, *C. litteratus*, *C. marmoreus*, *C. miliaris*, *C. sponsalis*, *C. ventricosus*) illustrating the clustering of 117 sequences (blasted with Tox-Prot database) which encompasses the numerous vermivorous and the rarest (in this dataset) molluscivorous predatory diets. The annotation colour from the inner circle to the outer is the following: organ, diet, species and Blast target.

The conotoxins demonstrated in Tree A and B are mostly from the M-1 branch (CM31_CONAE [A], CM35_CONTS [A], CM315_CONBU [A], CM31_CONLE [B], CM302_CONTS [A,B], CM32_CONPE [A,B], CM32_CONLE [A,B], CM33_CONLT [A,B], CM38_CONMR [A,B], CM34_CONLT [A,B], M3A_CONTE [A,B], CM3E_CONMR [A,B], CM3F_CONMR [A,B], CM31_CONPE [A,B], M32_CONTE [A,B], CM36_CONPL [A,B], and M3H_CONTE [A,B]), with additional three from the M-3 branch (CM31_CONQU [A], CM11_CONTS [A,B], and CM336_CONVE [A,B]), and one without attributed branch (CM401_CONAE [B]). The Mini-M group is characterized for containing fewer than 22 residues. It shows unparalleled structural diversity and cysteine connectivity, exhibiting distinct features such as unique patterns of disulphide bond connectivity, variability in loop sizes, and specialized 3D folding structures, even within the same branch. Until now, only members of the M-4 and M-5 branches have identified target receptors. Although little is understood about the biological receptors associated with Mini-M peptides, it is known they target receptors distinct from those of the Maxi-M group [56]. Given that most species here studied are vermivorous, this could mean that the mysterious target receptors of the conotoxins from the Mini-M group are more prominently present in worms, and studying them could reveal more about the nature of these conotoxins. Briefly, the 21 blast targets from the first two phylogeny trees belong to the most diverse and one of the most ubiquitous, yet enigmatic of the conotoxin superfamilies.

Both trees have 9 species, but while *C. betulinus*, *C. coronatus*, *C. ebraeus*, *C. litteratus*, *C. miliaris* and *C. sponsalis* are common in A and B, *C. ermineus*, *C. rattus* and *C. virgo* are exclusive to Tree A, while *C. judaeus*, *C. marmoreus* and *C. ventricosus* are exclusive to Tree B. The species from Tree A are all vermivorous, with one exception, *C. ermineus*, which is piscivorous (Figure 4). The lack of inclusion of the rest of the piscivorous species besides *C. ermineus* is debated in section 4.2. On the other hand, Tree B includes vermivorous species with the only molluscivorous, *C. marmoreus* (Figure 5). This tree (B) is constructed with 17 blast targets, having 2 less and sharing 15 with the previous one (A).

Overall, these two phylogeny trees show the most shared, conserved sequences of conotoxins among vermivorous and molluscivorous species, which have potentially been lost for most of the piscivorous species during evolution.

Because neither Tree A nor B have incorporated all 3 diets, criteria B was carried out and the result was a single cluster with 53 branches. A total of 10 cone species are identified in this Tree C: *C. betulinus*, *C. coronatus*, *C. ermineus*, *C. literatus*, *C. marmoreus*, *C. miliaris*, *C. quercinus*, *C. tribblei*, *C. ventricosus* and *C. virgo* (Figure 6). All seven different blast targets on this tree are plasma-membrane zinc ion-binding proteins, contrasting with the previous trees where the blast targets are actual conotoxins. From the eight vermivorous snails the blast targets very widely, from another mollusc, an owl limpet (V3ZVY9_LOTGI), to polychaete sea worms (R7UA33_CAPTE, R7UQB9_CAPTE), shelled brachiopods (A0A2R2MJ28_LINUN), and interestingly even a coral species (A0A2B4RU89_STYPI). Then, for the one molluscivorous species, *C. marmoreus*, we got a metalloendopeptidase originating from a fellow marine invertebrate mollusc, a blue mussel (A0A8S3SD11_MYTED). Finally, for the piscivorous *C. ermineus* the blast target is from a white-tufted-ear marmoset (U3E5A6_CALJA). Although remaining a metalloendopeptidase, its vertebrate origin mirrors the before mentioned affinity of the fish-eating cone snails' venom to vertebrates, showcasing the reason of its deadliness in humans, for example. Altogether, this phylogeny tree shows the regulatory peptidase sequences expressed in the venoms of cone snails and conserved in all feeding habits.

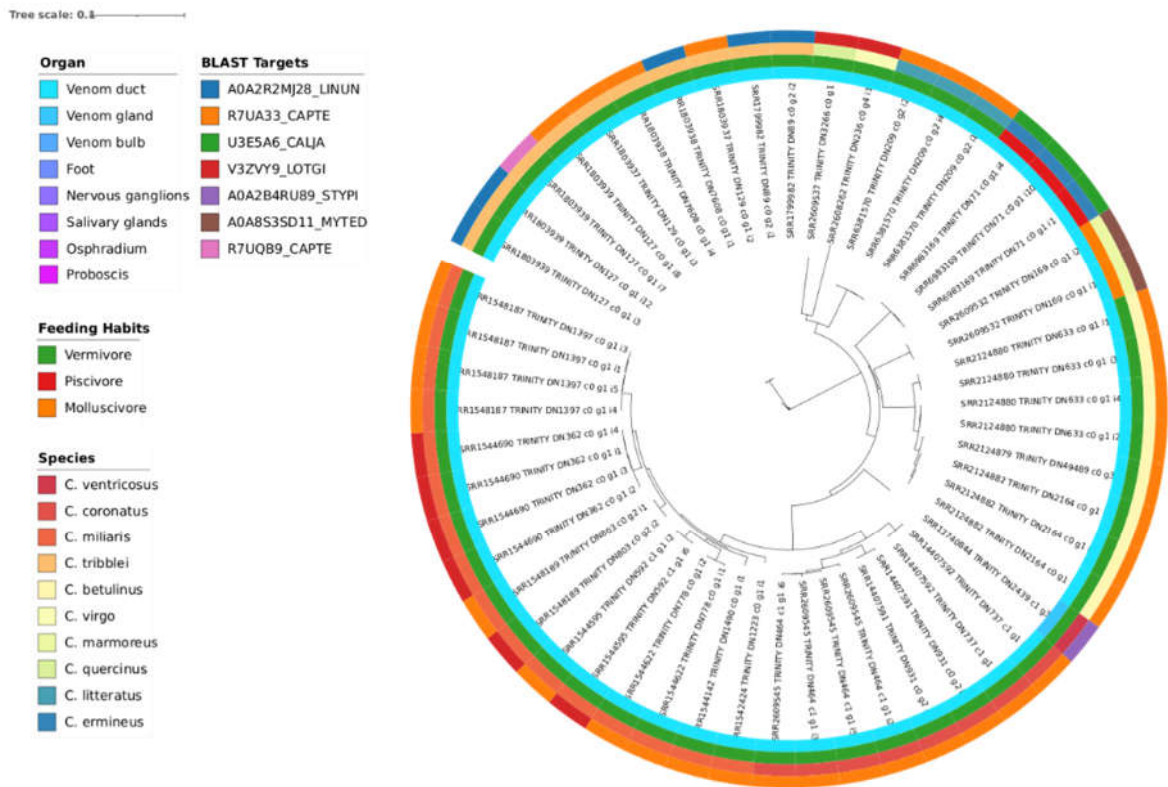


Figure 6. Phylogeny Tree C of 10 *Conus* species (*C. betulinus*, *C. coronatus*, *C. ermineus*, *C. literatus*, *C. marmoreus*, *C. miliaris*, *C. quercinus*, *C. tribblei*, *C. ventricosus*, *C. virgo*) illustrating the clustering of 53 sequences (blasted with UniProt Trembl database) which encompasses all three different feeding habits. The annotation colour from the inner circle to the outer is the following: organ, diet, species and Blast target.

3.4. AMP Prediction Results

3.4.1. AMP Potential of the Shared Venom Transcripts

A total of 16 AMP sequences belonging to 7 potential AMP kinds were observed, as resumed in Table 2. The first AMP sequence comes from a *C. betulinus* matching with a pulmonary surfactant-associated protein B precursor. Next, another single sequence from a *C. ebraeus* shows similarity with a ribonuclease. From *C. ermineus*, 4 sequences show anti-microbial potential, all related to an antimicrobial NK-lysin precursor. Finally, the last AMP comes from 10 different sequences of venom-related transcriptomes of 2 different *Conus* species which show similarity with the human ACE2 protein. Of these 10 sequences, coming from the venom duct we found 4 sequences from 2 transcriptomes of *C. literatus*, 2 sequences from 2 transcriptomes of *C. miliaris*, another 2 sequences but from just 1 transcriptome of *C. imperialis*, and finally 1 sequence from a transcriptome of *C. ventricosus* and another from a *C. quercinus*. As these AMP sequences are not related to conotoxins, they are likely from other kinds of venom peptides.

Table 2. Summary of AMP prediction from the sequences with the 14 chosen exclusively venom-shared annotations.

Data index	File	Species (diet)	Abundance	Data base hit	Description
AMP_00195	SRR2124881	<i>C. betulinus</i> vermivorous	1	AGANDLCQECEDIVHLL TKMTKEDAFQDTIRKFL QEC DILPLKLLVPRCRQV LDVYLPLVIDYFQGQIKP KAICSHVGLC	Pulmonary protein B precursor
AMP_03456	SRR6983168	<i>C. ermineus</i> piscivorous	1	GLICESCRKIIQKLEDMV GPQPNEDTVTQAASQVC DKLKILRGLCKKIMRSFL RRISWDILTGKKPQAICV DIKICKEKT	Antimicrobial peptide NK-lysin precursor
AMP_03457	SRR6983168		1	GLICESCRKIIQKLEDMV GPQPNEDTVTQAASQVC DKLKILRGLCKKIMRSFL RRISWDILTGKKPQAICV DIKICKEKTGLI	
AMP_04409	SRR6983168		1	GYFCESCRKIIQKLEDMV GPQPNEDTVTQAASQVC DKLKILRGLCKKIMRSFL RRISWDILTGKKPQAICV DIKICKE	
AMP_21444	SRR6983168	<i>C. ermineus</i> piscivorous	1	YFCESCRKIIQKLEDMVG PQPNEDTVTQAASQVCD KLKILRGLCKKIMRSFLRR ISWDILTGKKPQAICVDIK ICKE	Ribonuclease, RNase A family, 8
AMP_05220	SRR17653518	<i>C. ebraeus</i> vermivorous	1	KPKDMTSSQWFKTQHV QPSPQACNSAMSIINKYT ERCKDLNTFLHEPFSSVAI TCQTPNIACKNSCKNCH QSHGPMSTMGELTSGK YPNCRYKEKHLNTPYIVA CDPPQQGDPGYPLVPVH LDKVV	
AMP_19944	SRR6381569 SRR6381570	<i>C. literatus</i> vermivorous	4	EEQAKTFLDKFNHEAED LFYQSSLASWNYNTNITE E (See Appendix D for more transcripts sharing similarities with ACE protein)	ACE2
	SRR1542424 SRR1544142	<i>C. miliaris</i> vermivorous	2		
	SRR12186679	<i>C. imperialis</i> vermivorous	2		
	SRR13740844	<i>C. ventricosus</i> vermivorous	1		
	SRR2609537	<i>C. quercinus</i> vermivorous	1		

However, since cone snails are specialized in hunting either fish, molluscs or worms, the transcriptomic data was grouped according to predatory diet while still sorted by transcriptome assembly size (Figure 8). Fortunately, there are representative species of all feeding habits, although just one molluscivorous. Starting with the mollusc diet, the results are very modest, but provide a starting context for what could be expected with such assembly size. Next, the results show a very clear peak of AMPs predicted in fish eating *Conus* transcriptomes, from 83 in the start, to 128 then reaching the high towering numbers of 432 and 456 in transcriptomes of *C. consors*. The following feeding habit to the right, the vermivorous one, possesses the greatest number of exemplars (55 transcriptomes). Despite sudden heights and drops, a trend of increasing AMP number along growing assembly size can be noticed, especially in the transcriptomes larger than 60 Mb, although not reaching the same levels verified in the fish-eating diet.

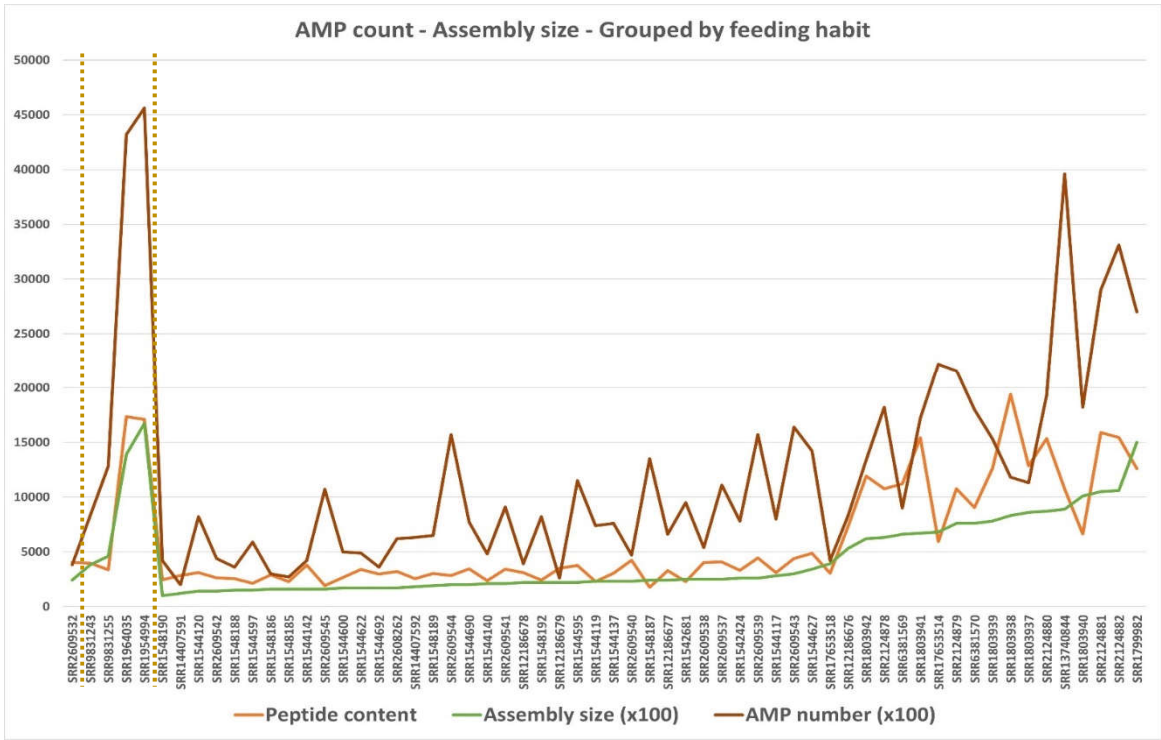


Figure 8. The linear graph illustrates the general trend of peptide content (orange) and AMP number (brown) per transcriptome grouped by feeding habits and in crescent order of assembly size (green); the vertical dark-yellow dotted lines separate the feeding habit grouping, from left to right: molluscivorous in the left, piscivorous in between and vermivorous to the right.

4. Discussion

4.1. Symbiosis in Venom Organs

Sorocarp morphogenesis (GO:0031288), negative regulation of asexual reproduction (GO:1903665), and – admittedly to a lesser extent – positive regulation of chemorepellent activity (GO:1903669) are indications of some kind of sporulating bodies existing specifically in the venom organs of all 20 *Conus* species analysed. These annotations are clearly unrelated to any biological process or molecular function of cone snails, indeed being described in the Gene Ontology knowledgebase for amoebas (e.g., *Dictyostelium discoideum*) and fungal organisms.

The suspicion of cone snails hosting some kind of symbiosis specifically in the venom organs is not new. Indeed, in 2009 a close symbiotic relationship in the venom duct of three cone snail species was revealed [39]. After exposing the existence of a thriving symbiotic actinomycete community, its results suggested that certain species of symbionts may be hosted in all species of cone snail, while other groups may be specific to their host. In 2017, another study rather unequivocally demonstrated that “cone snails have a distinct and consistent bacterial community” [40]. This research found diverse gene sequences closely matching *Stenotrophomonas* bacteria in all venom ducts examined. Such results were based on a worldwide variety of *Conus* species and showed that the 16S rRNA sequences retrieved represented specific symbiotic bacteria habiting the venom duct precisely where bioactive venom peptides are produced. More recently in 2023, it has been study hypothesised the existence of cone snail symbiosis with a seemingly new worm species found inside the egg capsules of *C. ermineus* [41]. Among several discoveries, the novel annelid species, *Dimorphilus oophagus* sp. nov. is contemplated as possible symbiont on account of isolated specimens not surviving more than two days in captivity. While the reason could be related to a normal short life cycle (also seemingly typical in species of the Dinophilidae family), it could also be the case that these specimens cannot survive outside its host egg masses because of their hypothetical parasite, commensal, or mutualist symbiotic nature.

Conclusively, studying more cone snails is essential to test, confirm and explore these symbiotic hypotheses. However, the discovery of GO:0031288, GO:1903665, and GO:1903669 in all transcriptomes of venom-related tissues assumes paramount importance in serving as compelling evidence to support the proposition of symbiotic microorganisms in the venom apparatus of all *Conus* species.

4.2. Predation Impact on Venom Evolution

The present study focuses on 20 cone snail species, a selection representing approximately 2% of the estimated total genus diversity (exceeding 800 recognized species). Despite the limited sampling size, there is a comprehensive representation all major phylogenetical clades, providing a good overall coverage of this genus' evolutionary diversity. This is supported by comparing our phylogeny tree (elaborated for the 20 species using 16S, see Appendix F) with a more extensive phylogenetic analysis involving over 350 *Conus* (elaborated with barcode sequences, including 12S, 16S and COI) [57].

The first clustering experiment (following criteria A) failed to generate a single tree, instead producing two trees (A and B, Figure 4 and 5, respectively) with an impressive array of toxins belonging to the most diverse and ubiquitous superfamily of conotoxins. This result prompted the lowering of the overall similarity threshold (criteria B), resulting in the creation of Tree C (Figure 6). The reason for criteria A to consistently produce two trees instead of one can only be understood when the predatory diet of each species is considered, as Tree A clusters together vermivorous and a piscivorous species and Tree B clusters the vermivorous and molluscivorous species. While B and even C trees connected harmoniously the vermivorous and molluscivorous species, there was a general failure to incorporate piscivorous species. Interestingly, the only piscivorous cone snail in both trees A and C is *C. ermineus*, not *C. consors* or *C. magus*, and we argue that is not a coincidence. Based on genetic studies performed in this work (see Appendix F) and by our group in the past, *C. ermineus* does not seem to share any immediate cladistical link with other piscivorous cone snails, in fact being more closely related to vermivorous cone snails [57]. Moreover, despite being classified as piscivore, *C. ermineus*' feeding habit remains somewhat confusing [58]. Its inclusion in the cluster with other vermivorous species indicates that it might still be in the process of piscivory specialization, convergently evolving a piscivorous diet similar to other fish-eating *Conus* [59]. A surprising recent discovery showed that even established piscivorous cone snails (like *C. magus*) feed on sea worms in its early juvenile age, further illustrating the higher complexity of the piscivorous diet and possibly increasing variation in venom gene expression during the development stage [60]. Following the second clustering attempt resulting in the C tree, there is a noticeable complexity drop in the blasted targets: from entire conotoxins in the first two clusters to smaller, ubiquitous zinc proteases in the last cluster with the three types of diets. The absence of more specialized piscivorous species in Tree A combined with the complexity drop of the Tree C points to a significant transcriptomic gap between the venoms of piscivorous species and the others. Indeed, the first clusters seem to indicate a whole group of genes conserved in the vermivorous and molluscivorous diets but mostly absent from the piscivorous one, excepting the transitory species *C. ermineus*. The reason for this might be connected with greater predatory advantages for worm-hunting rather than fish-hunting, resulting in specialization through the gradual loss of these genes (or their silencing after juvenile stage). The phenomenon of gene loss in venom evolution is described in various fellow venomous species, such as the previously mentioned rattlesnakes [34–36]. The notable distance in the transcriptomic content of piscivorous cone snail' venoms might also have direct implications in the previously mentioned deadliness to vertebrates and especially to humans [61]. Another important factor in the evolution of piscivorous venoms is the reported recruitment of the presumably more powerful defensive toxins into the offensive predatory venom arsenal [62]. This behaviour could exacerbate significantly the difference between the piscivorous species and the others, helping explain the absence of more specialized fish hunting snails in our results.

In essence, these results suggest that venom evolution and broad variability is directly influenced by preying selections. Recently, an article expanded on the issue of venom evolution shifts in these marine snails by analysing the structure of about 3,000 toxin genes and the overall venom composition from 42 species of cone snails [63]. Such research reached the conclusion that prey-specialization is a crucial factor driving the variability of the venom, especially in piscivorous species. Thus, the feeding habit emerges as a crucial clue to understand the seeming irregular variability in these gastropods' venom.

4.3. Biomedical Findings in *Conus* Venoms

A prediction of 88 potential AMPs (see Appendix E) is an indication that the *Conus* venom might be an interesting source of these peptides, but the results show a numerically biased distribution towards piscivorous species and larger transcriptomes (data size above 60 Mb) (Figure 8). Comparatively, vermivorous species also show an increasing number of potential AMPs in larger transcriptomes, but the trend shows inconsistencies and the overall number of AMPs is lower than in fish-eating *Conus*. As there is only one mollusc-eating species present in this study, any picture regarding the reliability of AMP finding in *Conus* with this specific diet is difficult to draw. However, greater numbers of piscivorous and molluscivorous species (and transcriptomes) need to be evaluated for a stronger (in the former) and statistically meaningful (in the latter) AMP prediction.

The reason for the greater number of AMPs predicted in piscivorous species might be connected to their large and good-quality transcriptomes (above 100 Mb) (see Appendix G). Indeed, two out of four of these transcriptomes possess the highest and third highest transcriptome sizes of the whole dataset (167 and 139 megabytes, respectively). However, the deadlier nature of fish-eating *Conus* venoms to vertebrates could also be influencing the results, as AMPfinder's database more prominently integrates peptides which directly target other vertebrates (75% of the AMPs in the database of AMPfinder originate from animals that hunt other vertebrates) [61,64,65]. This fact may influence the counting of AMPs as this tool's. This may eventually create a bias for similar stats of peptide length, electrical charge, hydrophobicity and other characteristics such as structural features. Thus, as the predatory venom from molluscivorous and vermivorous species is harmless to humans, it could be safer to develop clinical applications products such as AMPs from the conopeptides of non-fish-eating species.

4.4. ACE2 similarity

We identified a short transcriptomic sequence with AMP potential (see Table 2) exhibiting remarkable similarity to the human angiotensin-converting enzyme 2 (ACE2) protein, plus other sequences showing some analogy with ACE protein from a variety of animals such as the crown-of-thorns starfish and the Golden apple snail (see Appendix D). Although significantly shorter than the full-length ACE2 gene (36 amino acids in our result to 805 amino acids of the ACE2 gene), the sequence homology suggests a potential structural convergence or similar receptor-binding domains. The ACE2 protein is a critical component in human physiology and virology, being a key enzyme in the renin-angiotensin system (RAS) [66]. However, this finding is particularly intriguing given the fact that ACE2 is the primary cellular receptor for SARS-CoV-2, the virus responsible for COVID-19 [67,68].

As previous studies demonstrate the furin cleavage site of the viral Spike protein and certain conotoxins share similarities, we hypothesize that the short sequences we found may encode protein fragments that mimic a portion of the ACE2 receptor binding domain, which could potentially interfere with viral attachment and entry into host cells [69,70]. Further studies should be conducted on the functional significance of these short sequences. Bioinformatic analysis can help identify conserved domains and motifs, while protein structure prediction can provide insights into the three-dimensional conformation and potential binding sites. Functional validation experiments, including in vitro binding assays and viral infection models, are crucial to determine if these fragments can

indeed antagonize the Spike protein and inhibit viral entry. The potential implications of this discovery extend beyond our current understanding of cone snail venom. These short sequences, if proven to interfere with viral-ACE2 interactions, could serve as promising scaffolds for the development of novel therapeutic agents against SARS-CoV-2 and potentially other viruses that utilize ACE2 for host cell entry. Furthermore, the structural similarity between these fragments and ACE2 could be harnessed for the design of biosensors for rapid and specific viral detection.

5. Conclusions

In conclusion, our novel strategy in the study of *Conus* species combined a broad transcriptomics approach which encapsulated all publicly available data with a biomedical prediction model, contributing to further comprehend and unveil hidden potentials behind the complex predatory venom of these fascinating marine gastropods. In summary, our findings not only shed light on venom evolutionary traits and variability, but also hold promise for the development of novel antimicrobial therapeutics.

This research revealed an intrinsic link between venom evolution and predatory diet by showing that the main conopeptides conserved in vermivorous and molluscivorous species are absent in specialized piscivorous species. Their absence manifests the evolutionary distance and the probably more sophisticated nature of the piscivorous species venom compared to the others. Furthermore, transcriptomic evidence of symbiotic relationships within the venom organs was detected with the surprising findings of shared transcripts related to the symbiont's biology. On account of their presence in all venom transcriptomes, this discovery points to the existence of ubiquitous symbiotic microorganisms in cone snails. Secondly, by applying a comparative bioinformatic model to the venom-transcriptomes, a total of 88 potential AMPs were predicted. This suggests that the *Conus* marine venoms may present a viable source of AMPs, much sought for to counter the current growing challenge of drug resistant trend inflammatory pathogens. In addition, the finding of ACE2-like sequences opens an exciting door for future research.

For forthcoming investigations on the biomedical potential of conopeptides, the methodology should include machine learning prediction models beyond the comparative database bioinformatic method presented here. The employment of such *in-silico* mechanisms would both increase the range and confidence of the prediction result. In addition to AMPs, prediction of potential anti-cancer peptides could be targeted as well, given the identical methodology between the two objectives. Regarding *Conus* venom variability and evolution, we propose the standardization of a feeding habit context, not only in *Conus*, but also in other groups encompassing closely related species that exhibit different feeding habits.

Author Contributions: Conceptualization, J.M., Y.Z. and A.A.; methodology, J.M. and Y.Z.; software, J.M. and Y.Z.; validation, A.A. and L.H.; formal analysis, J.M. and Y.Z.; investigation, J.M. and Y.Z.; resources, A.A. and L.H.; data curation, J.M. and Y.Z.; writing—original draft preparation, J.M. and Y.Z.; writing—review and editing, A.A., L.H., and Y.Z.; visualization, A.A., L.H., J.M. and Y.Z.; supervision, A.A.; project administration, A.A.; funding acquisition, A.A. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original transcriptomic data supporting the retrieved results is publicly available.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ACE2

Angiotensin-Converting Enzyme 2

AMP(s)

Anti-Microbial Peptide(s)

Mb(s)

Megabyte(s)

PTM(s)

Post-Transcriptional Modification(s)

Appendix A

Table A1. The 14 GO IDs chosen from the 29 shared exclusively in the venom-related transcriptomes.

GO ID	GO Category	GO Term
GO:0003081	Biological process	Regulation of systemic arterial blood pressure by renin-angiotensin
GO:0003084	Biological process	Positive regulation of systemic arterial blood pressure
GO:0019882	Biological process	Antigen processing and presentation
GO:0030212	Biological process	Hyaluronan metabolic process
GO:0031288	Biological process	Sorocarp morphogenesis
GO:0071577	Biological process	Zinc ion transmembrane transport
GO:0097067	Biological process	Cellular response to thyroid hormone stimulus
GO:0140206	Biological process	Dipeptide import across plasma membrane
GO:1903052	Biological process	Positive regulation of proteolysis involved in protein catabolic process
GO:1903665	Biological process	Negative regulation of asexual reproduction
GO:1903669	Biological process	Positive regulation of chemorepellent activity
GO:0016532	Molecular function	Superoxide dismutase copper chaperone activity
GO:0016671	Molecular function	Oxidoreductase activity; acting on a sulphur group of donors; disulphide as acceptor
GO:0031545	Molecular function	Peptidyl-proline 4-dioxygenase activity

Appendix B

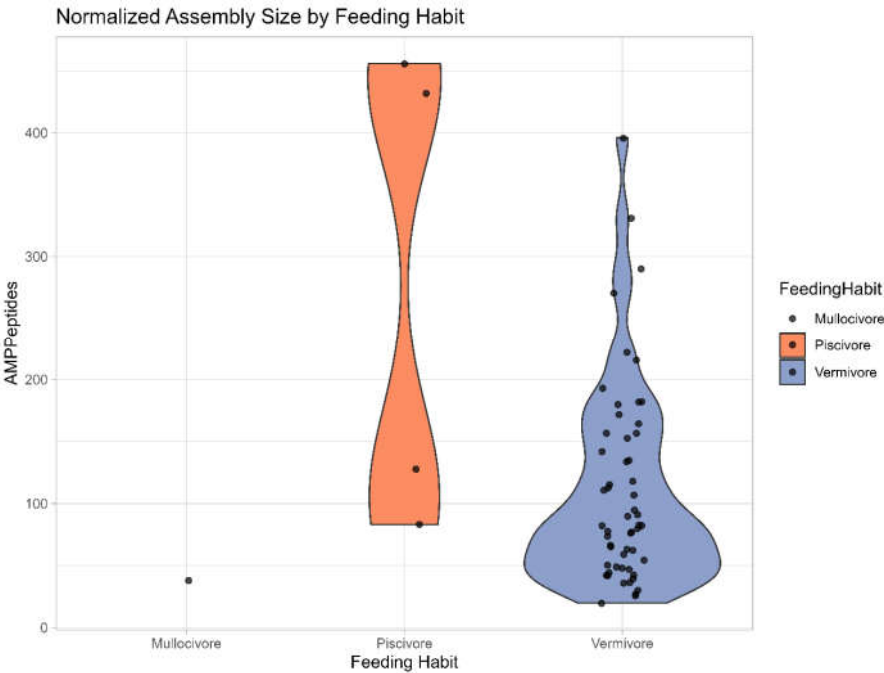


Figure A1. Violin Plot generated with R software, showing the distribution of AMPs grouped by feeding habit (molluscivore on the left, piscivore in the middle and vermivore on the right).

Appendix C

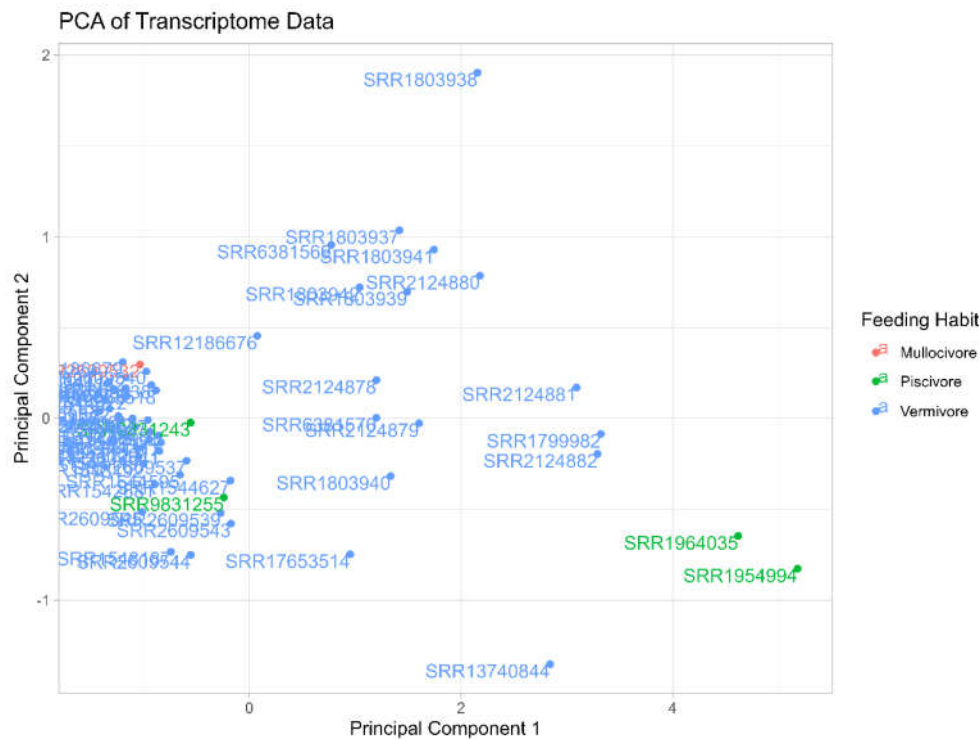


Figure A2. Principal Component Analysis (PCA) plot demonstrating the different distributions of transcriptomes according to their AMP number and coloured by predatory diet (pink for molluscivore, green for piscivore and blue for vermivore).

Appendix D

Table A2. 11 Conus transcriptomic sequences with great similarity with the Angiotensin-Converting Enzyme gene.

Sequences	Percentage of Identity
>SRR1544119_TRINITY_DN22969_c0_g1_i1	
CGGCACTGAAGGACGAGAACAAAGTGGCACGGTTTAACGAGCTAGTGGCCAAGATG TCGGAGATCTACAGCACCGCTCAAGTGTGCTTTACGGAAGGCAACTGTATCTCCCTG GATCCAGACCTCAAACGGCTCTTTGAACACAGCAGAACTACACATTGCTGACTGAA GCCTGGGAGCTGTGGAGAAAGGCCACTGGAGGAAAAATGAAAGCCCTGTACGAGG AGTATGTTGAGCTGGCGAACGAAGGCGTTCAGGAACTGGGTTTCAACGACATGGGA GAGTACTGGCGAGATGACTACGAAGACGAA	51,5% LOC106073291 Biomphalaria glabrata (Bloodfluke planorb) (Freshwater snail)
>SRR1544120_TRINITY_DN2907_c0_g1_i1	
TTGCCAACGCCACCCAGCGGCGCCTGCTGAAGCAAATCCGCAAGATCGGCACGGCG GCACAGAAGGACGAGAACAAAGTGGCACGGTTTAACGAGCTAGTGGCCAAGATGTC	50% LOC106073291 Biomphalaria glabrata

GGAGATCTACAGCACCGCTCAGGTGTGCTTTACGGAAGACAACGTATCTCCCTGGA TCCAGACCTCAAACGGCTCTTTGAACACAGCAGAACTACACATTGCTGACTGAAGC CTGGGATCTGTGGAGAAAGGCCACTGGAGGGAAAATGAAAGCCCTGTACGAGGAGT ATGTTGAGCTGGCGAACGAAGGCGTTCAGGAACTGGGTTTCAACGACATGGGAGAG TACTGGCGAGATGACTACGAAGACGAA	(Bloodfluke planorb) (Freshwater snail)
>SRR1544137_TRINITY_DN16819_c0_g1_i1 CGGCACTGAAGGACGAGAAACAAAGTGGCACGGTTTAACGAGCTAGTGGCCAAGATG TCGGAGATCTACAGCACCGCTCAAGTGTGCTTTACGGAAGGCAACTGTATCTCCCTG GATCCAGACCTCAAACGGCTCTTTGAACACAGCAGAACTACACATTGCTGACTGAA GCCTGGGAGCTGTGGAGAAAGGCCACTGGAGGAAAAATGAAAGCCCTGTACGAGG AGTATGTTGAGCTGGCGAACGAAGGCGTTCAGGAACTGGGTTTCAACGACATGGGA GAGTACTGGCGAGATGACTACGAAGACGAA	51,5% LOC106073291 Biomphalaria glabrata (Bloodfluke planorb) (Freshwater snail)
>SRR1544140_TRINITY_DN13380_c0_g1_i1 AGAACCTGGCGAGGAGCTGGCTACAGAAATACAACCAAGAGCACAAAGACATTTTC TCCAAGTCCTCAGAAATGACCTGGAACTACGCTACCAACGTCACGGACGAAATTCAA CAAAAACAAGTGAATGCAGAGCTGCAGGTGGCGCAATGGCAACAGGAGAAGGCAG CAGAAGTGGAACATTACGACTGGGAGCATTTTTCCGACAGCAGTCTTGTACGTCAGT TCCGTTTTGCGAGGAATATAGGCACGTCTGCCATG	52,9% C0Q70_02791 Pomacea canaliculata (Golden apple snail)
>SRR1544595_TRINITY_DN5707_c0_g1_i1 CTGAGGCTGGACACAACTGAGGGCCATGCTGTCCAAAGGATCGTCTGAGGTGTGG ACAGTACCATTCCAGGCCCTGACAGGACAGACCAAGATGAGCGCACAATCACTGAT CCAGTACTTCCAGCCCCTCATGGACTACCTGGAGCAGTACACCAAGGACCACGGCGT GGAGGTTGGGTGGAAGGAGGAGTGTTC	61,9% C0Q70_16356 Pomacea canaliculata (Golden apple snail)
>SRR1544600_TRINITY_DN9395_c0_g1_i1 TCGTTTTTCATGGCAGACGTGCCTATATTCCTTGCAAAACGGAACGTACGTACAAGAC TGCTGTCCGAAAAATGCTCCCAGTCGTAATGTTCCACTCCGCTGCCTTCCTCTGTTGC CATTGCGCCACCTGCAGCTCTGCATTCACTTGTTTTGTGATTTTCGTCGGTGACGTT GGTAGCGTAGTTCAGGTCATTTCTGAGGACTTGGAGACAATGTCTTTGTGCTCTTGG TTGTATTTCTGTAGCCAGCTCCTCGCCAGGTTCTC	56,3% C0Q70_02791 Pomacea canaliculata (Golden apple snail)
>SRR1544692_TRINITY_DN15875_c0_g1_i1 TCGTTGCTCAGCGCCACGAACTCCTCGTAATCGCTCTTCATCAGAGGCCCTGTGACGT CACGCCACTCCTTCCACGCCATCAGCAGTTCGTCATAGTCACGCGATGACGCCATCA GTTTGGTCAGTTCAGGATCCAGATTCAAAATGGCCCCGGTCTTTGGATCCTTCACTTT AGCTTTGGCGTAGATACCTTCAATGGTCGACTGTAGCTCTTTAGCTCTTTCAGTTTAG TTTCGTTTTTCATGGCAGACGTGCCTATATTCCTTGCAAAACGGAACGTACGTACAAG ACTGCTGTCCGAAAAATGCTCCCAGTCGTAATGTTCCACTCCGCTGCCTTCCTCTGTT	55,8% C0Q70_02791 Pomacea canaliculata (Golden apple snail)

GCCATTGCGCCACCTGCAGCTCTGCATTCACTTGTTTTGTTGATTTTCGTCCGTGACG TTGGTAGCGTAGTTCACAGGTCATTTCTGAGGACTTGGAGATAATGTCTTTGTGCTCTTT GTTGTATTTCTGTAGCCAGCTCCTCGCCAGGTTCTC	
>SRR1544692_TRINITY_DN2117_c0_g1_i1	68,3% LOC110975981
TCCTCACTCCAGCCAACGGGCTGGCCGGCGTTCTGCTCCTCCAGCCAATCCTGAAGC GGCCTGAAGTACTCCAGCAGCGGCCTCACGTCCATGTGTCTGGTGCCCGTGATCTGCT CCAGGGCCTC	Acanthaster planci (Crown-of-thorns starfish)
>SRR6983166_TRINITY_DN44676_c0_g1_i1	56,8% C0Q70_02791
ACAGCACCGCTCGAGTGTGCTTTACGGAAGACAACGTATTCCCCTGGACCCGGACG TCAAACGGCTCTTTGAACACAGCAGAAACTACACATTGCTGGCTGAAGCCTGGGATC TGTGGAGAGAGGCCACTGGAGGAGAAATGAAAGCCCTGTACGAGGAGTATGTTACAG CTGGGAAACGAAGGAGTTCAGGAACTGGGTTTCAACGACATGGGAGAGTACTGGCG AGACGAGTATGAAGACGAA	Pomacea canaliculata (Golden apple snail)
>SRR6983168_TRINITY_DN6724_c0_g2_i1	49% C0Q70_02791
CGGACAGCGAGGCAGTGGAAGCCTTCTTGAGACGCACGACAAGGAGACTAAGAA GAAGCATGAAAAGTACGAGATTCTGTCTGGAATCACGAAACCAATATCACCGACT ACAATCAGGAGCTGAAGGTCAACTACAGCGTAGAGATGTCAGAATTTGCTAAAGAG CACGCCAGGCAGTCGGCCATGTTTGACCTTGATCACCTTGCCAACGCCACCCAGCGG CGCCTGCTGAAGAAAATCGGCAAAATCGGCACGGCGGCACAGAAGGACGAGAACA AAGTGGCACGGTTTAACGAGCTGGTGGCCAAGATGTCGGAGATCTACAGCACCGCTC GAGTGTGCTTTACGGAAGACAACGTATTCCCCTGGACCCGGACGTCAAACGGCTCT TTGAACACAGCAGAAACTACACATTGCTGGCTGAAGCCTGGGATCTGTGGAGAGAG GCCACTGGAGGAGAAATGAAAGCCCTGTACGAGGAGTATGTTACGCTGGGAAACGA AGGCGTTCAGGAACTGGGTTTCAACGACATGGGAGAGTACTGGCGAGACGAGTATG AAGACGAAAATCTGCAAGAAGAGTTAGCGGCCCTGATGGAACAGTCCGTCCTTTGT ACGTGAAGCTCCAC	Pomacea canaliculata (Golden apple snail)
>SRR6983169_TRINITY_DN17529_c0_g1_i1	51% C0Q70_02791
TTCTGTCTGGAATCACGAAACCAATATCACCGACTACAATCAGGAGCTGAAGGTCA ACTACAGCGTAGAGATGTCAGAATTTGCTAAAGAGCACGCCAGGCAGTCGGCCATG TTTGACCTTGATCACCTTGCCAACGCCACCCAGCGGCGCCTGCTGAAGAAAATCGGC AAAAATCGGCACGGCGGCACAGAAGGACGAGAACAAAGTGGCACGGTTTAACGAGC TGGTGGCCAAGATGTCGGAGATCTACAGCACCGCTCGAGTGTGCTTTACGGAAGACA ACTGTATTCCCCTGGACCCGGACGTCAAACGGCTCTTTGAACACAGCAGAAACTACA CATTGCTGGCTGAAGCCTGGGATCTGTGGAGAGAGGCCACTGGAGGAGAAATGAAA GCCCTGTACGAGGAGTATGTTACGCTGGGAAACGAAGGCGTTCAGGAACTGGGTTTC AACGACATGGGAGAGTACTGGCGAGACGAGTATGAAGACGAA	Pomacea canaliculata (Golden apple snail)

Appendix E

Table A3. AMPfinder software prediction results; each AMP is unique, attesting to its AMP database ID; the Protein ID columns show the function of the sequence hit obtained and the Abundance columns show the number of each particular AMP; the colours represent the tissue origin of each AMP: green for AMPs coming from the venom-related transcriptomes, yellow for venom-unrelated transcriptomes and red for those present in both groups.

Protein ID	AMP ID	Abundance	Protein ID	AMP ID	Abundance
P83578 IKP1_PHYSA	AMP_11771	7	P20160 CAP7_HUMAN	AMP_14282	20
P61095 SFH1_SEGFL	AMP_15699	4	P0DKT2 TU92_GEMSO	AMP_25599	20
Q90WJ8 AJL2_ANGJA	AMP_00722	3	P20160 CAP7_HUMAN	AMP_14283	16
P39060 COIA1_HUMAN	AMP_14664	3	P00738 HPT_HUMAN	AMP_04692	15
P01038 CYT_CHICK	AMP_23459	2	P05484 O17A_CONMA	AMP_18775	11
P0C2D2 OXLA_CRODC	AMP_00139	1	P05484 O17A_CONMA	AMP_18778	11
Q9JHY3 WFD12_MOUSE	AMP_02769	1	P05484 O17A_CONMA	AMP_18779	11
P22887 NDKC_DICDI	AMP_09422	1	P05484 O17A_CONMA	AMP_18780	11
P22355 PSPB_RAT	AMP_00195	1	P05484 O17A_CONMA	AMP_18781	11
Q29075 NKL_PIG	AMP_03456	1	P06702 S10A9_HUMAN	AMP_09443	6
Q29075 NKL_PIG	AMP_03457	1	P06702 S10A9_HUMAN	AMP_11387	6
Q29075 NKL_PIG	AMP_04409	1	P05484 O17A_CONMA	AMP_27220	6
Q29075 NKL_PIG	AMP_21444	1	P05484 O17A_CONMA	AMP_27221	6
Q8TDE3 RNAS8_HUMAN	AMP_05220	1	P05484 O17A_CONMA	AMP_27222	6
A0A7I2V2E9 A0A7I2V2E9_HUMAN	AMP_19944	10	P83952 WAPA_OXYMI	AMP_04982	4
P00974 BPT1_BOVIN	AMP_10608	76	B5G6G7 WAPB_OXYMI	AMP_04983	4
I2G9B4 VKT_MACLN	AMP_15720	76	P83240 NDB31_PANIM	AMP_02393	2
P20160 CAP7_HUMAN	AMP_04915	62	P83240 NDB31_PANIM	AMP_02394	2
P20160 CAP7_HUMAN	AMP_04916	62	P83240 NDB31_PANIM	AMP_02395	2
P20160 CAP7_HUMAN	AMP_04917	62	A0A0C4G5K0 NDB3_HE TSP	AMP_02396	2
P20160 CAP7_HUMAN	AMP_04918	60	P82427 WTX1E_NEOGO	AMP_02397	2
P08311 CATG_HUMAN	AMP_04624	56	P0DJ02 NDB49_HETPE	AMP_08335	2
P86810 OXLA_SIGCA	AMP_11257	50	A0A0C4G5K0 NDB3_HE TSP	AMP_17099	2
P86810 OXLA_SIGCA	AMP_11258	50	P83240 NDB31_PANIM	AMP_22673	2
Q4JHE1 OXLA_PSEAU	AMP_00142	48	P83240 NDB31_PANIM	AMP_22674	2
F8S0Z5 OXLA2_CROAD	AMP_00224	48	P83240 NDB31_PANIM	AMP_22676	2
P04284 PR06_SOLLC	AMP_07416	45	P83240 NDB31_PANIM	AMP_22677	2
P04284 PR06_SOLLC	AMP_10197	45	P13487 KAX11_LEIHE	AMP_01368	1
P81382 OXLA_CALRH	AMP_00135	44	A9XE60 KBX11_MESEU	AMP_02691	1
Q6TGQ8 OXLA_BOTMO	AMP_00137	44	P82656 NDB21_HOFAZ	AMP_03049	1
Q90W54 OXLA_GLOBL	AMP_00138	44	F1AWB0 NDB27_VAEME	AMP_03190	1

Q6STF1 OXLA_GLOHA	AMP_00422	44	F1AWB0 NDB27_VAEME	AMP_03191	1
Q6STF1 OXLA_GLOHA	AMP_00423	44	P83239 NDB23_PANIM	AMP_03236	1
Q6TGQ9 OXLA1_BOTJR	AMP_08879	43	P83313 NDB24_OPICA	AMP_03238	1
Q9U8W7 TL5B_TACTR	AMP_01246	42	P83313 NDB24_OPICA	AMP_03239	1
B5AR80 OXLA_BOTPA	AMP_00127	41	P83314 NDB2S_OPICA	AMP_04307	1
B5AR80 OXLA_BOTPA	AMP_00128	41	A0A0C4G489 NDB2_HET SP	AMP_04308	1
Q9U8W8 TL5A_TACTR	AMP_10191	41	P0C2F4 KBX3_HETLA	AMP_04346	1
P00734 THRB_HUMAN	AMP_09738	39	P56972 KBX3_PANIM	AMP_04348	1
P00734 THRB_HUMAN	AMP_09739	39	Q0GY40 KBX3_HOFGE	AMP_04390	1
P20160 CAP7_HUMAN	AMP_14281	29	Q5WR03 KBX31_OPICA	AMP_05482	1
Q1PHZ4 VM3B1_BOTJR	AMP_01520	27	P13487 KAX11_LEIHE	AMP_10082	1
Q6IWZ0 OXLA_APLCA	AMP_01042	26	P0C2F4 KBX3_HETLA	AMP_17928	1
Q6IWZ0 OXLA_APLCA	AMP_01043	26	A9XE60 KBX11_MESEU	AMP_23221	1

Appendix F

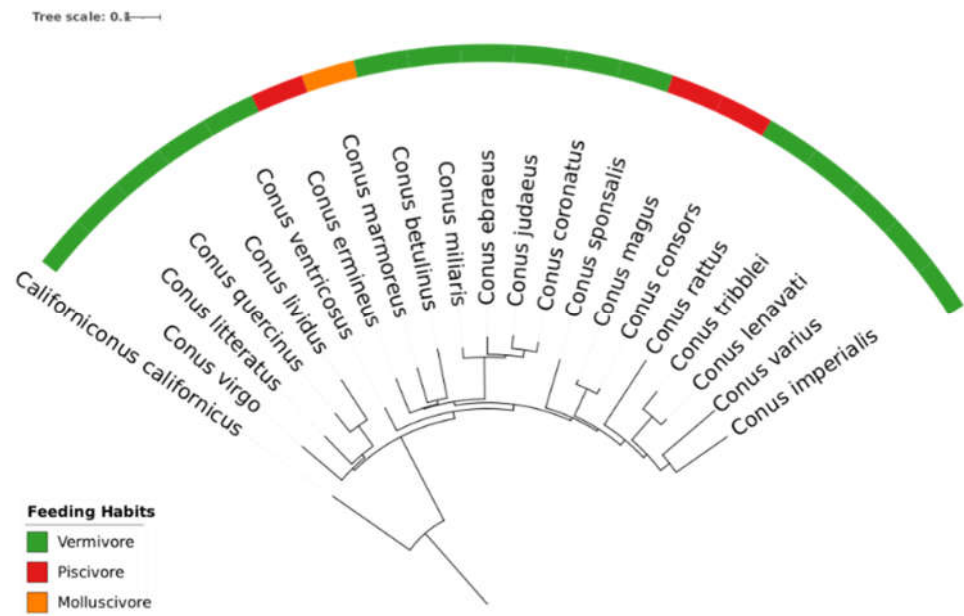


Figure A3. Phylogeny tree of 21 *Conus* species using two rRNA 16S genes for each species. The colours represent feeding habits: green for vermivorous, red for piscivorous, and orange for molluscivorous; *Conus californicus* in the root serves as the reference outgroup species.

Appendix G

Table A4. BUSCO assessment performed against BUSCO’s Metazoa set for all 76 transcriptome assemblies.

Species and Tissue	Completed - Total	Complete - Single Copy	Complete - Duplicated	Fragmented	Missing
C. miliaris Venom duct	110	94	16	75	769

C. miliaris Venom duct	122	102	20	85	747
C. coronatus Venom duct	122	108	14	74	758
C. miliaris Venom duct	128	109	19	98	728
C. miliaris Venom duct	128	110	18	98	728
C. miliaris Venom duct	142	119	23	116	696
C. miliaris Venom duct	153	129	24	108	693
C. miliaris Venom duct	153	129	24	106	695
C. miliaris Venom duct	166	135	31	118	670
C. miliaris Venom duct	161	136	25	98	695
C. miliaris Venom duct	174	138	36	113	667
C. miliaris Venom duct	164	140	24	138	652
C. coronatus Venom duct	166	144	22	122	666
C. miliaris Venom duct	174	145	29	95	685
C. miliaris Venom duct	180	151	29	133	641
C. miliaris Venom duct	176	151	25	104	674
C. miliaris Venom duct	191	161	30	135	628
C. miliaris Venom duct	191	162	29	135	628
C. miliaris Venom duct	190	163	27	111	653
C. consors Foot	244	163	81	129	581
C. coronatus Venom duct	192	166	26	128	634
C. sponsalis Venom duct	203	167	36	127	624

C. miliaris Venom duct	196	173	23	115	643
C. miliaris Venom duct	210	176	34	163	581
C. miliaris Venom duct	230	189	41	133	591
C. imperialis Venom duct	220	190	30	114	620
C. virgo Venom duct	210	191	19	150	594
C. imperialis Venom duct	227	192	35	162	565
C. coronatus Venom duct	222	193	29	114	618
C. miliaris Venom duct	241	196	45	148	565
C. ebraeus Venom duct	229	200	29	180	545
C. imperialis Venom duct	240	207	33	148	566
C. ermineus Venom duct	256	214	42	111	587
C. lividus Venom duct	259	215	44	189	506
C. ermineus Venom duct	256	219	37	184	514
C. imperialis Venom duct	251	222	29	125	578
C. marmoreus Venom duct	247	222	25	151	556
C. miliaris Venom duct	268	224	44	184	502
C. ermineus Venom duct	271	228	43	110	573
C. ebraeus Venom duct	286	232	54	86	582
C. rattus Venom duct	282	234	48	186	486
C. quercinus Venom duct	267	237	30	161	526

C. ermineus Venom duct	301	249	52	130	523
C. varius Venom duct	297	250	47	163	494
C. ermineus Venom duct	294	251	43	175	485
C. tribblei Venom duct	307	253	54	281	366
C. tribblei Venom duct	313	256	57	264	377
C. magus Venom duct	315	258	57	106	533
C. tribblei Venom duct	318	267	51	256	380
C. lenavati Venom duct	343	274	69	262	349
C. lenavati Venom duct	334	274	60	207	413
C. ermineus Venom duct	356	274	82	104	494
C. magus Venom duct	344	279	65	113	497
C. lenavati Venom duct	365	284	81	253	336
C. betulinus Venom duct	355	286	69	263	336
C. ermineus Venom duct	364	287	77	103	487
C. betulinus Venom duct	388	301	87	268	298
C. ermineus Venom duct	390	314	76	116	448
C. judaeus Venom duct	421	337	84	123	410
C. litteratus Venom duct	453	357	96	230	271
C. litteratus Venom duct	500	371	129	218	236
C. ventricosus Foot	471	382	89	121	362
C. imperialis Venom duct	460	385	75	170	324

C. ventricosus Foot	544	424	120	120	290
C. ermineus Venom duct	574	429	145	133	247
C. consors Nervous ganglions	771	446	325	94	89
C. consors Venom duct	750	458	292	90	114
C. ventricosus Venom gland	584	463	121	124	246
C. betulinus Venom duct	568	464	104	208	178
C. consors Osphradium	836	465	371	65	53
C. consors Salivary glands	694	480	214	130	130
C. betulinus Venom duct	662	485	177	154	138
C. consors Proboscis	826	489	337	63	65
C. consors Venom bulb	751	489	262	99	104
C. betulinus Venom duct	600	517	83	205	149
C. tribblei Venom duct	667	542	125	165	122

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