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Evolution of Vanadium-Binding Protein (Vanabin) Gene Structure in Phlebobranchia and Aplousobranchia Ascidian Lineages

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Ascidians within order Phlebobranchia can accumulate extremely high levels of vanadium (V) in their blood cells. Several V-related proteins, including V-binding proteins (vanabins), have been isolated from V-accumulating ascidians. Vanabins were first isolated from a V-rich ascidian, *Ascidia sydneiensis samea*, belonging to Phlebobranchia. In this study, we searched for vanabin gene homologs in all available public databases containing tunicate genomic data and the draft genome database of *A. sydneiensis samea* constructed in this study. We found one to six vanabin genes per genome among all available genomes of Phlebobranchia species. We also found a vanabin gene in the genome of an Aplousobranchia species. Thus, we identified vanabins in seven ascidian species, and aligned these according to the C–Xn–C pattern common to the vanabin core domain. Based on a molecular phylogenetic tree derived from 18S ribosomal DNA and natural resistance-associated macrophage protein sequences, we propose a model for the evolution of vanabin gene structure via the following pathway: acquisition of ancestral vanabin by the common ancestor of Phlebobranchia and Aplousobranchia, followed by vanabin gene duplication; nonsynonymous mutation, insertion, and positive selection to create amino acid variation among vanabins; and extension of the vanabin gene cluster. The selective force acting within this pathway may have been V toxicity under high-V conditions.

Key words: ascidians, evolution, gene structure, metal accumulation, metal-binding protein

INTRODUCTION

Ascidians, also known as tunicates or sea squirts (Chordata, Urochordata, Ascidiacea), can accumulate extremely high concentrations of vanadium (V) (Henze, 1911; Michibata et al., 1986). Species belonging to order Phlebobranchia accumulate a maximum of 350 mM V, corresponding to approximately 10^7 times the concentration of V ions in natural seawater (Cole et al., 1983; Collier, 1984). In natural seawater, V ions in the +5 oxidation state [V(V)] are taken up by ascidians, reduced to the +3 oxidation state [V(III)], and stored in the vacuoles of coelomic blood cells called vanadocytes (Ueki et al., 2015; Ueki and Michibata, 2011).

Many genes involved in V accumulation and reduction have been identified in ascidians through biochemical and molecular biological methods, mainly in the V-rich ascidian species *Ascidia sydneiensis samea*, which can accumulate up to 12.8 mM V in its blood cells. V-binding proteins called vanabins were first isolated from ascidian blood cells via ion exchange chromatography (Kanda et al., 1997). After the cloning of Vanabin1 and Vanabin2 in *A. sydneiensis samea* (Ueki et al., 2003), four additional vanabins were identified through metal ion affinity chromatography, ion exchange chromatography, expressed sequence tag (EST) analysis (Yamaguchi et al., 2004; Yoshihara et al., 2005), and RNA-seq analysis (Adi et al., 2022) of the same ascidian species. Vanabins have also been identified in other V-rich ascidian species. The genome database for the ascidian *Ciona intestinalis* type A (*Ciona robusta*) (Dehal et al., 2002) has yielded five vanabins to date (Trivedi et al., 2003); and our own EST analysis of intestinal tissue from *Ascidia gemmata* yielded two vanabins, AgVanabin1 and AgVanabin2 (Samino et al., 2012). Thus, vanabins are unique among V-related proteins in that they have been identified only in V-rich ascidians.

All known vanabins have 18 conserved cysteine residues, which form nine disulfide bonds to create a bow-shaped structure (Hamada et al., 2005), except for VanabinX, which lacks two of these cysteines (Adi et al., 2022). Some

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of these disulfide bonds are reversible via redox reactions, allowing vanabins to act as V(V)-reductases (Kawakami et al., 2009). In previous studies, we proposed a model in which V(V) ions are readily reduced to V ions via +4 oxidation [V(IV)] by vanabins in the cytoplasm, and V(IV) ions are stabilized by vanabins (Kawakami et al., 2009; Kitayama et al., 2013; Yamamoto et al., 2014). Thus, vanabins act as both V reductases and V chaperones.

The proteins most similar to vanabins in extant organisms of the ascidian lineage are metallothioneins, which are small, cysteine-rich proteins ubiquitous among nearly all organisms. Mammalian metallothioneins consist of 61 or 62 amino acid polypeptides and contain 20 cysteines, which coordinate seven–10 divalent metal ions (Marikar, 2022). Invertebrate metallothioneins differ slightly from those in mammals. Insect metallothioneins are classified into six clades, of which clade A is the shortest, with an average length of 38 amino acids, and clade F is the longest, with an average length of 62 amino acids (Luo et al., 2020). Among ascidians, one metallothionein (CiMT-1), a protein composed of 39 amino acids, was reported from *Ciona intestinalis* (Franchi et al., 2011), and two from *Herdmania curvata* (metallothionein A-like AY314949 and metallothionein B-like AY314939) were directly submitted to the National Center for Bioinformation (NCBI) database; the latter are putatively composed of 64 and 41 amino acid proteins, respectively. It is possible that vanabins evolved from small, metal-binding proteins such as metallothioneins.

The first published tunicate genome (*Ciona intestinalis* type A, i.e., *C. robusta*; [Dehal et al., 2002]) was later updated based on chromosome-level mapping (Satou et al., 2008) and de novo genome assembly (Satou et al., 2019). However, since these studies, several other tunicate genomes have been published, and many tunicate genomes have become publicly available. In the present study, we developed an evolutionary model of vanabin gene structure, consisting of a possible evolutional pathway based on sequence information for putative vanabins from public databases. We collected all publicly available tunicate genome data to conduct a comprehensive search for vanabin genes. Then, we compared domain patterns among all conserved vanabin amino acid sequences, as well as the number and size of each vanabin gene cluster.

MATERIALS AND METHODS

Database search and domain pattern and sequence comparisons

We obtained tunicate genomic and transcriptomic data from the NCBI (https://www.ncbi.nlm.nih.gov/), Ascidian Network for *In Situ* Expression and Embryological Data (ANISEED; https://aniseed.fr/) (Brozovic et al., 2018), Ghost (https://ghost.zool.kyoto-u.ac.jp/) (Satou et al., 2005), and Oikobase (http://oikoarrays.biology.uiowa.edu/Oiko/) (Danks et al., 2013) databases in June and July 2024. Draft genome data for *A. sydneiensis samea* was obtained and published in this study as described in the next section, and transcriptome data for this species has already been published (Adi et al., 2022). Raw data obtained from NCBI were assembled using the Velvet *de novo* assembler (Zerbino and Birney, 2008). The data sources are listed in Supplementary Table S1.

Vanabin protein amino acid sequences have been published for three species: *A. gemmata* (Samino et al., 2012), *A. sydneiensis samea* (Ueki et al., 2003; Yamaguchi et al., 2004; Yoshihara et al., 2005; Adi et al., 2022), and *C. robusta* (Trivedi et al., 2003). Using the amino acid sequences of these known vanabins, we conducted a BLAST+ search of the NCBI and ANISEED databases (Camacho et al., 2009) based on a threshold of $e < 0.005$. The resulting amino acid sequences (see Supplementary Table S2) were classified by their domain structures and cysteine repeat patterns based on visual inspection.

Draft genome sequencing for *A. sydneiensis samea*

Adult *A. sydneiensis samea* individuals were collected at Kojima Port, Okayama, Japan, and cultivated in an aquarium under flowing natural seawater at the Marine Biological Laboratory, Seto Inland Sea Carbon-neutral Research Center, Hiroshima University, Hiroshima, Japan. They were fed regularly with diatoms (*Chaetoceros* sp.). Genomic DNA was extracted from ascidian sperm using the phenol/chloroform extraction method. All sequencing libraries were constructed according to the manufacturers' standard protocols. Briefly, on an Illumina platform, polymerase chain reaction (PCR)-free paired-end libraries were prepared using an Illumina TruSeq DNA PCR-Free LT Library Prep Kit (FC-121-3001; Illumina, San Diego, CA, USA). Five mate-pair libraries were prepared using a Nextera Mate Pair Library Prep Kit (FC-132-1001; Illumina). Shotgun and mate-pair sequence reads were assembled using Platanus v1.2.4 (Kajitani et al., 2014). Scaffolds longer than 1000 bp were selected as draft genome data (5368 contigs; median length, 390,744 bp; total length, 121,037,280 bp; see Supplementary Table S1). Scaffold sequences were deposited in the DNA Data Bank of Japan database (accession nos. BAAHWS010000001–BAAHWS010005368). Preliminary annotations were done by BLAST+ search using known proteins including Vanabins based on a threshold of $e < 0.005$.

Phylogenetic tree for natural resistance-associated macrophage proteins (Nramps) and vanabins

Nramp amino acid sequences were retrieved from the NCBI database as registered sequences or from the NCBI Sequence Read Archive. All obtained Nramp sequences are listed in Supplementary Table S3. Nramp and vanabin amino acid sequence alignments were generated using MAFFT v7 (Katoh et al., 2019). Molecular phylogenetic trees were constructed using the neighbor-joining method by MAFFT v7. Maximum Likelihood trees were constructed by IQ-TREE (Minh et al., 2020). Phylogenetic trees were drawn using Philo.io (https://phylo.io/). Tree nodes were evaluated based on 1000 bootstrap replicates (Felsenstein, 1985).

Vanabin homology and de novo modeling

Alphafold2 software was used to predict the protein structure of each vanabin (Jumper et al., 2021). Vanabin gene structures were visualized and aligned using PyMOL Molecular Graphics System v1.5.0.4 (https://pymol.org/2/). SWISS PDBViewer software (http://www.expasy. org/spdbv/) was used for surface electrical potential mapping (Guex and Peitsch, 1997).

Prediction of ancestral amino acid sequence

Ancestral amino acid sequence was predicted using IQ-TREE (Minh et al., 2020). The input sequences were those in Fig. 4. The prediction was done using default parameters.

RESULTS

Database search

We identified vanabins from all 10 Phlebobranchia species and one Aplousobranchia species, but not from Stolidobranchia species or any other tunicate groups (see Supplementary Table S1). Species from which vanabins were identified are listed in Table 1.

Table 1. List of ascidian species bearing vanabin genes.

order	family	species	genome/transcript data	number of vanabins
Phlebobranchia	Ascidiidae	*Ascidia gemmata*	EST analysis	at least 2 genes
Phlebobranchia	Ascidiidae	*Ascidia sydneiensis samea*	genome/RNA-seq	9 loci 6 transcripts
Phlebobranchia	Ascidiidae	*Ascidia mentula*	genome/RNA-seq	5 loci, 1 variable 20 transcripts
Phlebobranchia	Ascidiidae	*Ascidiella aspersa*	genome/RNA-seq	1 locus 4 transcripts
Phlebobranchia	Ascidiidae	*Phallusia mammillata*	genome/RNA-seq	at least 4 loci3 transcripts
Phlebobranchia	Ascidiidae	*Phallusia fumigata*	genome	multiple loci
Phlebobranchia	Cionidae	*Ciona intestinalis* type A (*C. robusta*)	genome new assembly old assembly	 7 loci 5 loci
Phlebobranchia	Cionidae	*Ciona savignyi*	genome	at least 2 loci
Phlebobranchia	Corellidae	*Corella eumyota*	genome/RNA-seq	1 locus 1 transcript
Phlebobranchia	Corellidae	*Corella inflata*	genome	Y1 loci

order	family	species	genome/transcript data	blast hit for vanabins
Aplousobranchia	Clavelinidae	*Clavelina lepadiformis*	genome/RNA-seq	3 loci, partial 1 transcript

Order Phlebobranchia

We collected genomic and transcriptomic data for six species of family Ascidiidae. Our previous EST analysis of *A. gemmata* identified two vanabins (Samino et al., 2012). The present study is the first to provide a draft genome sequence of *A. sydneiensis samea*. We identified seven vanabin genes from *A. sydneiensis samea* based on previously published transcriptomic data for blood cells of this species (Adi et al., 2022); however, one of these vanabins appeared to be partial or chimeric. Based on public NCBI data, we identified eight vanabins from *Ascidia mentula* and one from *Ascidia aspersa*. Four of the eight vanabins from *A. mentula* were short and appeared to be partial.

We collected genomic and transcriptomic data for two species of family Cionidae. *Ciona robusta* is the most extensively studied ascidian species. In addition to the five vanabins previously reported for this species (Trivedi et al., 2003), we identified two with a short core domain. Genomic data for *Ciona savignyii* yielded only two vanabins, perhaps due to the low assembly quality for this species. Therefore, we omitted the short sequences from *C. robusta* and the two sequences from *C. savignyii* from subsequent analyses.

Genomic and transcriptomic data for two *Corella* species (*Corella eumyota* and *Corella inflata*) were available from the NCBI. Each of these species had a single vanabin gene. Although the genome assembly for *C. inflata* was poor, the *C. eumyota* and *C. inflata* sequences were similar to each other, and the *C. eumyota* assembly was adequate. Therefore, we retained both vanabin sequences for subsequent analyses.

Order Stolidobranchia

We collected genomic and transcriptomic data for order Stolidobranchia based on two *Styela* species, two *Halocynthia* species, two *Molugula* species, two *Botrylloides* species, one *Botryllus* species, and one *Boltenia* species. We were unable to find vanabin genes in the genomes of any of these species.

Order Aplousobranchia

We collected genomic and transcriptomic data for order Aplousobranchia from one *Clavelina* species, one *Aplidium* species, two *Tridemnum* species, and one *Diplosoma* species. We identified three vanabin loci and one transcript from *Clavelina lepadiformis*; one appeared to have a complete core domain, whereas the remaining two were short. Therefore, we included the complete vanabin in subsequent analyses.

Other tunicates and lancelets

We also collected genomic and transcriptomic data for one *Salpa* species, two *Oikopleura* species, and two *Branchiostoma* species. We were unable to detect vanabin genes in the genomes of these species.

Amino acid sequence and pattern comparisons

Amino acid sequences predicted based on the genomic and transcriptomic data are listed in Supplementary Table S2. Given the broad variation in amino acid sequences, except for cysteines, we converted the sequences into C–Xn–C patterns. An alignment of C–Xn–C patterns for selected vanabins is shown in Fig. 1. The N-terminal domain consists of putative signal peptides, three of which have been proved experimentally: Vanabin1 and Vanabin2 (Ueki et al., 2003) and VanabinP (Yoshihara et al., 2005) in *A. sydneiensis samea*. The central domain is a cysteine-rich core domain; because vanabins typically possess 18 cysteines with regular intervals (termed C18 vanabins), we selected the 18 conserved cysteines according to visual inspection of their positions and intervals. We found novel vanabins with 16 cysteines (C16 vanabins) from *Corella* species; in these vanabins, the two middle cysteines were miss-

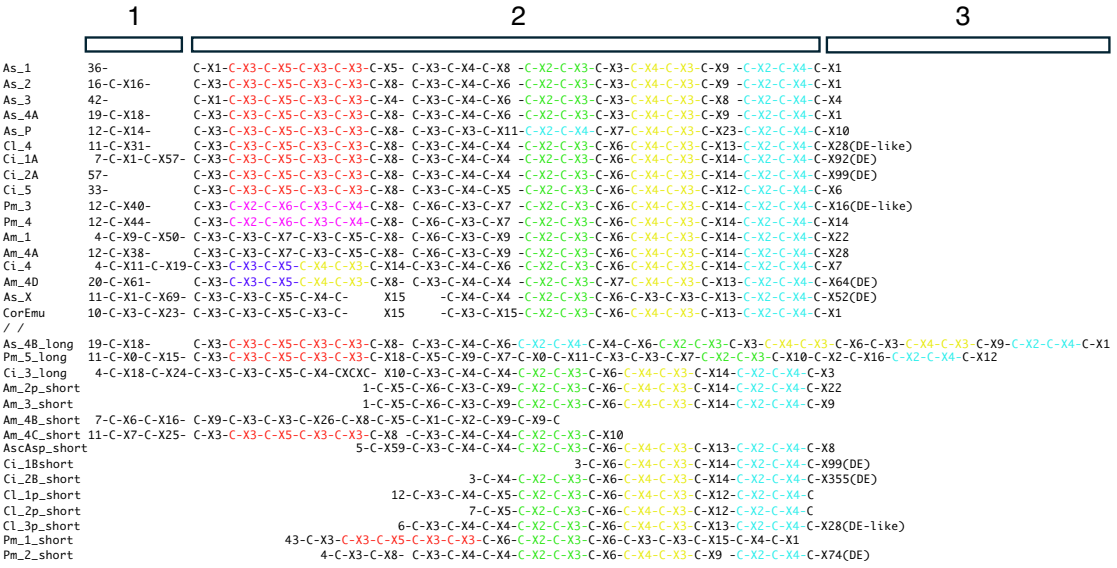


Fig. 1. Alignment of amino acid patterns among vanabins. (Left) Identification numbers for each vanabin (see Supplementary Table S2 online). (Right) Amino acid patterns, showing cysteines (C) and numbers (n) of amino acids other than cysteines (X). Some C–Xn–C patterns are colored to visualize similarities among vanabins. Upper panels show vanabins with typical C18 domain composition, including the N-terminal putative signal peptide, cysteine-rich core domain, and C-terminal stretch (containing D/E domains rich in aspartic acid and glutamic acids). Lower panels show vanabins with irregular or incomplete domain composition; these sequences were not used in our analysis.

ing, as was first reported for VanabinX from *A. sydneiensis samea* (Adi et al., 2022). Thus, the vanabin C-terminal domain is an acidic domain that contains many acidic amino acids (e.g., aspartic acid and glutamic acid), allowing the classification of C18DE and C16DE vanabins. These acidic domains were found in genera *Ascidia*, *Phallusia*, and *Ciona* within order Phlebobranchia, but not in the genera *Corella* or *Clavelina* within order Aplousobranchia.

Phylogenetic tree and C–Xn–C pattern evolution

Next, we constructed an evolutionary model for vanabin genes based on two molecular phylogenetic trees. The first tree was based on 18S ribosomal DNA (rDNA) sequences (Tsagkogeorga et al., 2009). Because the branching of the 18S rDNA sequence tree for ascidian families was obscure, we constructed a second tree using Nramp amino acid sequences. Nramps are V transporters (Ueki et al., 2011), and ascidians generally possess a single Nramp gene. The Nramp neighbor-joining tree matched well with the 18S rRNA tree, with high bootstrap support for the branching of each family (Fig. 2). ML trees were also constructed by the Maximum Likelihood method with two models, JTT and WAG, suitable for amino acid sequences, but the bootstrap support for branching among Ascidiidae, Cionidae, and Corellidae/Clavelinidae was not enough high (see Supplementary Figure S1). Therefore, we used the phylogenetic tree obtained by the neighbor-joining method to construct our evolutionary model of vanabin gene structure (Fig. 3).

In the model, seven species are clustered into two clades, one containing both Aplousobranchia (Clavelinidae) and Phlebobranchia (Corellidae), and the other containing only Phlebobranchia (Ascidiidae and Cionidae). This model predicts that the common ancestor for Phlebobranchia and Aplousobranchia possessed both a C18 vanabin and a C18DE vanabin. One vanabin disappeared in the lineage

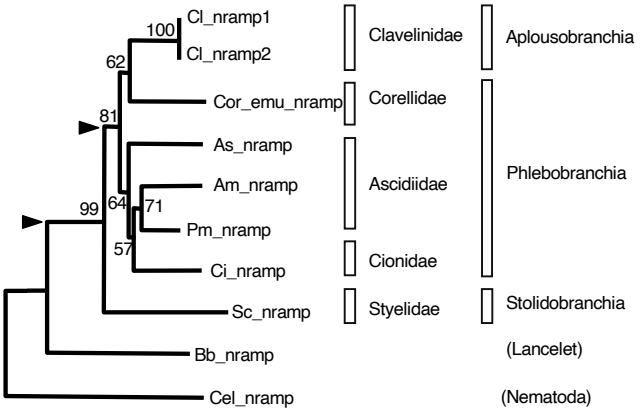
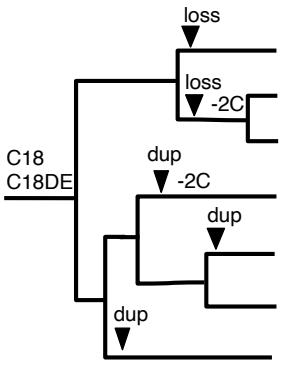


Fig. 2. Phylogenetic tree for natural resistance-associated macrophage proteins (Nramps). Eight species of five ascidian families were compared via the neighbor-joining method, using lancelet and nematode Nramps as an outgroup. Numbers at each branch indicate percent bootstrap support based on 1000 bootstrap pseudoreplicates. Arrowheads indicate nodes supported by Nramps but not by 18S rDNA sequence analysis. All sequences are listed in Supplementary Table S3.

leading to Corellidae and Clavelinidae, and extant *Corella* and *Clavelina* species possess one vanabin in their genomes. In the other lineage, leading to Ascidiidae and Cionidae, vanabins were duplicated tandemly to form a gene cluster. Some vanabins lost two cysteines to become C16 or C16DE vanabins. Within the *Ascidia* lineage, the gene cluster expanded through the incorporation of other genes.

Variation in amino acid sequence and structure

To gain insight into the functions of amino acids other than cysteines in vanabins, we compared amino acid



species	C16	C16 DE	C18	C18 DE	number of loci	range of cluster
Clavelina lepadiformis	0	0	0	1	1(+2)	(30k)
Corella eumyota	1	0	0	0		
Corella inflata	1	0	0	0		
Ascidia sydneiensis samea	0	1	5	0	9	270k
Ascidia mentula	0	0	2	1	6–11	30k
Phallusia mammillata	0	0	1	1	2(+3)	18k
Ciona intestinalis	0	0	3	2	5	8k

Fig. 3. Evolutionary model of vanabin domain composition and gene clusters. Left branch matches that of the Nramp phylogenetic tree shown in Fig. 2. Table at right summarizes the numbers of vanabins in each category (C16, C16DE, C18, and C18DE), numbers of loci in each gene cluster, and size range of each cluster. –2C, loss of two cysteines; dup, gene duplication; loss, gene loss.

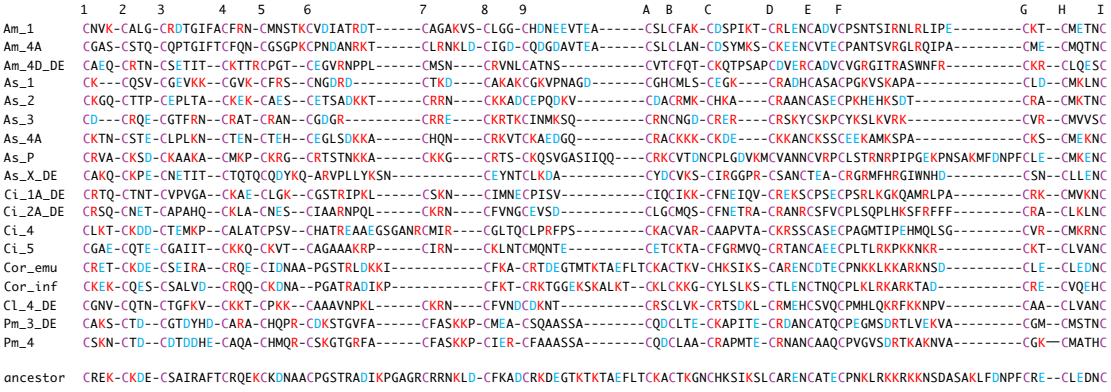


Fig. 4. Amino acid sequence alignment for the vanabins listed in Supplementary Table S2 and a putative ancestral sequence. Conserved cysteines are indicated in purple. Positively and negatively charged amino acids are indicated in red and blue, respectively.

sequences of vanabins with the typical domain composition. The alignment of amino acids sequences in the core domain is shown in Fig. 4. All cysteines were aligned in a manner consistent with those shown in Fig. 1; amino acids other than cysteines were set to follow each cysteine, and gaps were inserted to align cysteines.

The ancestral amino acid sequence was predicted by IQ-TREE (Fig. 4). Because we hypothesized that *Corella* species are closely related to the putative common ancestor, the predicted sequence was close to those of *Corella* vanabins; however, it had 18 cysteines because the prediction algorithm aimed to identify potential amino acid losses. The ancestral amino acid sequence was predicted to contain 20 lysines within the 128 amino-acid core domain (15.6%), which is reminiscent of Vanabin2 variants (Ueki et al., 2008). The most lysine-rich variant was 14MT, which contained 19 lysines within the 86-amino acid core domain (22.1%) (Ueki et al., 2008). The numbers and ratios of lysines are provided in Supplementary Figure S2, which shows the phylogenetic tree based on this alignment. *Corella* vanabins were separated from other vanabins, forming a clade. The other vanabins did not form clades for each species, but did for certain vanabin subtypes. Lysine-rich vanabins in *A. sydneiensis samea* (As_1, As_2, As_3, As_4A, and As_P) were positioned near the ancestral vanabin, whereas the 16DE VanabinX (As_X_16DE) was more distant. Therefore, this species may have lost lysines and cysteines after gene duplication. In *C. robusta*, 18DE (Ci_1A_DE and Ci_2A_DE) and C18 (Ci_4 and Ci_5) vanabins were duplicated independently. Thus, the diversification of amino acids other than cysteines appears to have occurred independently before or after gene duplication in each lineage.

Three-dimensional modeling

We modeled vanabin structure in *Corella* species using Alphafold2 software. Although the resulting structures (Cor_emu and Cor_inf, Fig. 5) were almost identical to that of *A. sydneiensis samea* Vanabin2 (As_2, Fig. 5), alignments of the predicted structures indicated that they were moderately conserved between *Ascidia* and *Corella* vanabins (root mean square deviation [RMSD] = 4.849 for As_2 and Cor_emu; RMSD = 5.236 for As_2 and Cor_inf), whereas those of *Corella* vanabins were more similar to each other (RMSD = 2.364).

The electrostatic surfaces of *Corella* vanabins were more positive than that of Vanabin2. Vanabin2 binds to V(IV) via amine nitrogen species in amino acid residues, such as lysines and arginines in the cysteine-rich core domain (Fukui

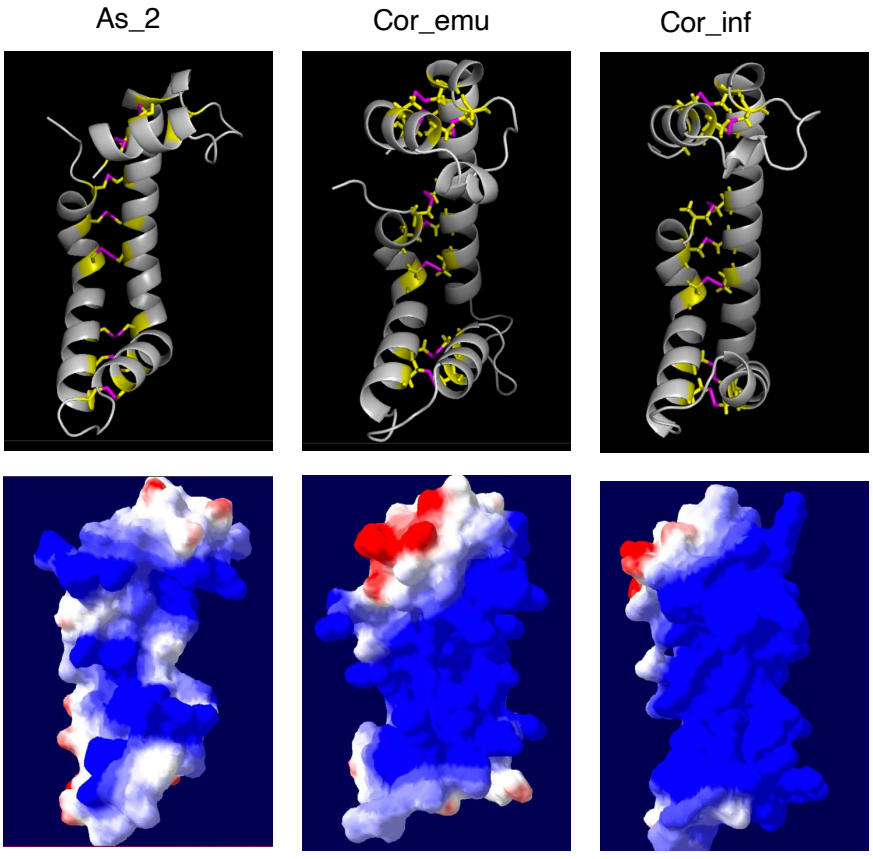


Fig. 5. Comparison of structural models for vanabins from *Ascidia sydneiensis samea* (Vanabin2, As_2; Supplementary Table S2), *Corella eumyota* (Cor_emu), and *Corella inflata* (Cor_inf). Upper panels show ribbon models; lower panels show surface electric potentials. Electric potential images were produced by SWISS PDBViewer using the following parameters: red, –2.2; white, 0.0; blue, 4.0. Positively and negatively charged amino acids are indicated in blue and red, respectively.

et al., 2003). Two V(IV) binding sites were identified through site-directed mutagenesis on Vanabin2 (K10, K24, K38, R41, and R42) (Ueki et al., 2009). *Corella* vanabins do not have lysines or arginines at these sites.

DISCUSSION

We searched genomic and transcriptomic data from all public databases containing tunicate genomic data, and identified vanabins from all 10 Phlebobranchia species and one Aplousobranchia species. We did not find vanabins from order Stolidobranchia or any other tunicate groups.

In extant organisms, metallothioneins are the proteins that most closely resemble vanabins. Metallothioneins are small, cysteine-rich proteins that are ubiquitous among extant organisms, but they are not rich in charged amino acids such as lysines or arginines. Based on a phylogenetic tree predicted from 18S rDNA sequences, the Stolidobranchia/Appendicularia and Phlebobranchia/Thaliacea/Aplousobranchia lineages first divided in tunicates (Tsagkogeorga et al., 2009). However, the relationship among Phlebobranchia, Thaliacea, and Aplousobranchia remains uncertain. The long branch of Aplousobranchia species disturbed the 18S phylogenetic tree, but when divergent species were removed, the slow evolving Clavelinidae made a clade with Phlebobranchia species, which is consistent with our phylogenetic tree. This is also supported by recent studies based on mitochondrial protein coding genes (Shenker et al., 2016) and nuclear conserved orthologous genes (Delsuc et al., 2018; Debiasse et al., 2020). Assuming that Thaliacea diverged first among these groups, the common ancestor of Phlebobranchia and Aplousobranchia must have obtained the first ancestral vanabin gene, which then duplicated to form a gene cluster. Nonsynonymous mutation, insertion, and positive selection occurred in each lineage to create amino acid variation among vanabins, and the extension of these gene clusters in one lineage led to the divergence of Ascidiidae. Although the type of natural selection involved in these processes remains unknown, the function of vanabin as a V(V) reductase and V(IV)-binding protein suggests that vanabins helped ancestral ascidians to survive in V(V)-rich environments. An example of a V-rich environment is the Lower Cambrian Black Shale of the Sansui vanadium deposits (Wu et al., 2021). In the model of Wu et al. (2021), vanadium was brought to the middle or lower area of the continental shelf by volcanic-hydrothermal activity in the early Cambrian, accumulated by clay materials and brought to shallow water areas by upwelling currents. We speculate that such V-rich occurrence may have facilitated the evolution of vanabin genes in ascidians. Although there are no studies on animals, enhancement of mutation rates and selection have been reported in bacteria in environments rich in heavy metal ions such as manganese (Sitompul et al., 2024).

Biochemical studies suggested that both C18 (Kawakami et al., 2009) and C16 (Adi et al., 2022) type Vanabins can reduce vanadium(V) to vanadium(IV) and the DE domain enhances the reductase activity (Adi et al., 2022). Since the C16-type Vanabin in *Corella* species was predicted to have a similar structure as C18 Vanabin, it must act as a vanadium(V)-reductase.

Despite the difficulty of gene structural modeling based on incomplete genomic information, we attempted to analyze the structures of the vanabin genes found in this study. Vanabins with somewhat reliable cluster structures, obtained from three different species, are illustrated in Supplementary Figure S3. Among these, five vanabins from *C. robusta* have already been reported (Trivedi et al., 2003); we confirm that a small gene cluster in this species contains five vanabin loci that are repeated tandemly in the same direction (see Supplementary Figure S3A). *Phallusia mammillata* has a similar cluster structure composed of five vanabin gene loci (see Supplementary Figure S3B). According to the phylogenetic tree generated in this study based on Nramp and vanabin amino acid sequences, the common ancestor must have had both C18 and C18DE vanabin genes. In the *Corella* lineage, one vanabin was lost or somehow modified. In other ascidians, vanabin genes were duplicated and diverged. In *A. sydneiensis samea*, the vanabin gene cluster was relatively large (see Supplementary Figure S3C) and contained many other genes (not shown). In *A. sydneiensis samea* (13 mM V in blood cells), all six vanabins are simultaneously transcribed in blood cells and appear to function synergistically in the cytoplasm (Adi et al., 2022). However, one of these vanabins, VanabinP, is secreted into blood plasma (Adi et al. 2022). Such functional and regional diversification within cells may account for the high V accumulation rates observed in *Ascidia* species (Fig. 6). It will be necessary to obtain genomic data for other *Ascidia* species such as *A. gemmata* (350 mM V in blood cells) and *A. ahodori* (60 mM V) to confirm this hypothesis.

Tandem gene duplication plays significant roles in genomic evolution and gene family expansion. Because the order and number of vanabin genes are not conserved among ascidians, tandem duplication must have occurred in each ascidian lineage, contributing to vanabin genetic diversity and adaptation in each lineage. In *A. sydneiensis samea* Vanabin2, the typical 18 cysteines form nine disulfide bonds (Hamada et al., 2005); thus, strong selective pressure must prevent amino acid substitutions of vanabin cysteine residues. Among amino acids other than cysteine, the diversification of amino acids appears to have occurred independently before or after gene duplication in each lineage, and the variation of ratios of lysine is intriguing. The predicted ancestral amino acid sequence contains a relatively higher ratio of lysines (15.6%) (Fig. 4), which may reflect the high lysine ratio in vanabins found in ascidian species with a single vanabin gene (14.0% for *C. eumyota* and 16.6% for *C. inflata*). In contrast, vanabins found in ascidian species with multiple vanabins tends to have a variety of lysine ratios between 3.2% and 19.8% (see Supplementary Figure S1). Since vanabins with low or high lysine ratio can bind to vanadium (IV) (Ueki et al., 2008), variation of the number of lysines may have occurred independently in each lineage as a result of weak selective pressure and thus it is not a criterion for suggesting phylogenetic relationships.

A transcriptomic approach (Delsuc et al., 2018), which included all major tunicate lineages and consisted of 258

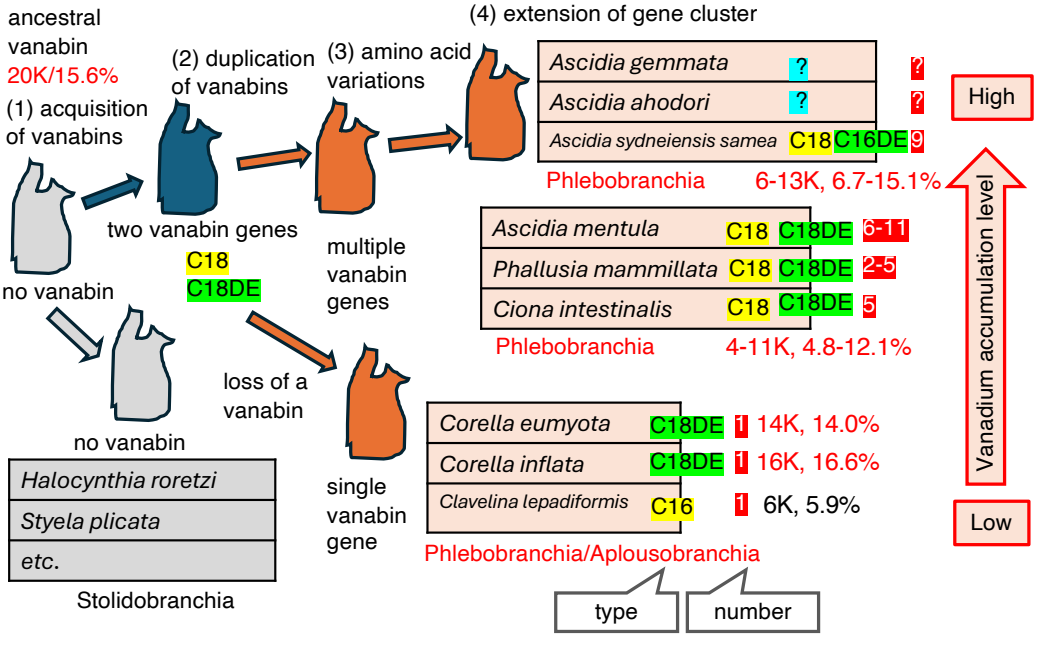


Fig. 6. Evolutionary model of vanabin gene structure. The proposed evolutionary pathway consists of the acquisition of ancestral vanabin in the common ancestor of Phlebobranchia and Aplousobranchia, followed by vanabin gene duplication, randomly occurring nonsynonymous mutation and insertion of amino acids, positive selection of lysine-rich vanabins, and vanabin gene cluster extension. C16, C18, C16DE, and C18DE indicate vanabin types. Numbers of gene loci are shown in boxes. The number (K) and percentage of lysines are shown for each ascidian group.

evolutionarily conserved orthologous genes from representative species, suggested that the divergence of stolidobranchs and the clade grouping thaliaceans + phlebobranchs + aplousobranchs occurred ca. 389 ± 32 million years ago (Mya). The divergence of Thaliaceans and phlebobranchs + aplousobranchs occurred 296 ± 44 Mya, and that of phlebobranchs and aplousobranchs occurred 259 ± 43 Mya. Combining our phylogenetic analysis results and those of Delsuc et al. (2018), vanabins must have been acquired by ascidians between these events (296 and 259 Mya), followed by vanabin gene diversification in each ascidian lineage.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

UT and NS designed the experiments. UT, TKA, and MF conducted the analyses. UT, TKA, MF, and NS wrote the manuscript.

SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: https://doi.org/10.2108/zs250091)

Supplementary Figure S1. Phylogenetic tree for natural resistance-associated macrophage proteins (Nramps). Eight species of five ascidian families were compared via the Maximum Likelihood method, using lancelet and nematode Nramps as an outgroup.

Supplementary Figure S2. Phylogenetic tree for vanabins (see Supplementary Table S2 online). A putative ancestral sequence (ancestor) was set as the root.

Supplementary Figure S3. Schematic illustration of vanabin gene clusters for **(A)** *Ciona robusta*, **(B)** *Phallusia mammillata*, and **(C)** *Ascidia sydneiensis samea*.

Supplementary Table S1. Genome and transcriptome data used in this study.

Supplementary Table S2. Amino acid sequences and C–Xn–C patterns of the vanabins used in this study.

Supplementary Table S3. Amino acid sequences of natural resistance-associated macrophage proteins (Nramps) used in this study.

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