## **Tunicates Illuminate the Enigmatic Evolution of Chordate** Metallothioneins by Gene Gains and Losses, Independent Modular Expansions, and Functional Convergences

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#### Abstract

To investigate novel patterns and processes of protein evolution, we have focused in the metallothioneins (MTs), a singular group of metal-binding, cysteine-rich proteins that, due to their high degree of sequence diversity, still represents a "black hole" in Evolutionary Biology. We have identified and analyzed more than 160 new MTs in nonvertebrate chordates (especially in 37 species of ascidians, 4 thaliaceans, and 3 appendicularians) showing that prototypic tunicate MTs are mono-modular proteins with a pervasive preference for cadmium ions, whereas vertebrate and cephalochordate MTs are bimodular proteins with diverse metal preferences. These structural and functional differences imply a complex evolutionary history of chordate MTs-including de novo emergence of genes and domains, processes of convergent evolution, events of gene gains and losses, and recurrent amplifications of functional domains-that would stand for an unprecedented case in the field of protein evolution.

Key words: metallothionein domains, modular proteins, Chordata, Tunicata, ascidians/thaliaceans/appendicularians, metallothionein evolution.

#### Introduction

Heavy metals such as zinc (Zn) and copper (Cu) are integral components of numerous proteins involved in multiple cellular functions (reviewed in Vallee and Falchuk [1993]; Maret [2013]; Vest et al. [2013]), whereas other metals such as cadmium (Cd) have no known biological functions and are highly toxic for living beings. Organisms have developed different molecular mechanisms that regulate the homeostasis of the physiological metals and counteract the harmful effects of the toxic ones. One of such mechanisms is based on an extremely diverse group of metal-binding proteins known as metallothioneins (MTs), which are engaged in the physiological control of metals operating as ion reservoirs, metal transporters and/or metal deliverers to target metalloproteins, but also in radical scavenging, oxidative stress protection, and antiapoptotic defense (reviewed in Capdevila et al. [2012]; Ziller and Fraissinet-Tachet [2018]).

Most MTs are low molecular weight (<100 amino acids) and cysteine-rich ( $\approx$ 15–30%) proteins. Their cysteine (Cys, C) residues are arranged in CxC, CC, and CCC motifs, whose number and distribution define functional domains. Many MTs have a bimodular organization made of two functional

domains, in which each domain enables the coordination of a number of metal ions through metal-thiolate bonds (reviewed in Blindauer [2014]). MTs have been classified in a stepwise gradation, from extreme Zn/Cd-thioneins to extreme Cu-thioneins depending on their metal-binding preference (Bofill et al. 2009; Palacios, Atrian, et al. 2011). There have been attempts to predict the metal preference of a given MT from the analysis of their non-Cys amino acids, and in the case of gastropod MTs, for instance, it has been proposed that the lysine-asparagine (K/N) ratio could be important for metal preference, so that a preponderance of K over N residues would be a feature of Cd selectivity, whereas a predominance of N over K would be typical of Cu-MTs (Perez-Rafael et al. 2014; Pedrini-Martha et al. 2020). Due to the high seguence and structural diversity of MTs, however, it is unclear whether the rule of K/N ratio applies to nongastropod MTs, and thereby, metal-binding assays are needed to experimentally determine the metal preference of newly discovered MTs.

Comparative analyses of the MTs across diverse species have been useful to better understand the function and evolution of these proteins, and to illustrate unusual modes of

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molecular evolution of these genes and proteins. MT research has exposed, for instance, pervasive events of internal sequence duplications (Tanguy and Moraga 2001; Palacios, Espart, et al. 2014; Iturbe-Espinoza et al. 2016; Jenny et al. 2016; Calatayud et al. 2018), de novo emergence of domains (Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021), and a high bias in the amino acid composition probably as a result of the 3D structural requirements for metal binding. MT research has also provided useful information for many other research fields. In the field of Evolutionary Ecology and Environmental Sciences, for instance, MTs are considered a good model to investigate the mechanisms by which different organisms-from protists and fungi to insects, mollusks, and mammals-have adapted to diverse metal bioavailability and other environmental stress conditions. These mechanisms include duplications of MT genes (Maroni et al. 1987; Adamo et al. 2012; Luo et al. 2020), increases in the levels of MT expression (Timmermans et al. 2005; Janssens et al. 2008, 2009; Costa et al. 2012; Catalan et al. 2016; de Francisco et al. 2018), and changes in metal specificity (Tio et al. 2004; Palacios, Pagani, et al. 2011; de Francisco et al. 2017; Dallinger et al. 2020; García-Risco et al. 2020). In the fields of Bioinorganic Chemistry and Metallomics, MTs have been used to analyze metal-protein interactions, analyzing the qualitative and quantitative characteristics of the metalbinding capacities of these proteins (Blindauer and Leszczyszyn 2010; Palacios, Pagani, et al. 2011; Beil et al. 2019) and by extension, of a broad range of metalloproteins (Waldron et al. 2009).

In this work, we aim to explore the MTs found in a large number of species of the three tunicate classes—Ascidiacea, Thaliacea, and Appendicularia-in order to investigate the intriguing origin and evolution of MTs in chordates. Tunicates (Tunicata subphylum, a.k.a. Urochordata) are the sister group of vertebrates, and together with basally branching cephalochordates form the phylum Chordata. Tunicates have undergone a rapid and "liberal" pattern of evolution in comparison with the "conservative" pattern of vertebrates and cephalochordates (Somorjai et al. 2018; Ferrández-Roldán et al. 2019), yielding a group of ecologically diverse filter-feeding marine animals-including planktonic and benthic specimens as well as solitary and colonial forms-, which have adapted to different conditions of metal bioavailability. In addition, the filtering lifestyle makes tunicates prone to accumulate metals from the seawater (Papadopoulou and Kanias 1977; Tzafriri-Milo et al. 2019; and references therein), and thereby, molecular mechanisms such as the MTs that regulate metal homeostasis and detoxification are physiologically relevant to them.

Our understanding of the MTs in tunicates is, however, scarce, limited to two MT sequences from the ascidian *Ciona robusta* (formerly C. *intestinalis*) and *Herdmania curvata* (Franchi et al. 2011), and two sequences from the appendicularian *Oikopleura dioica* recently described by our group (Calatayud et al. 2018). To overcome this situation, we have conducted an exhaustive survey of the MTs in tunicate databases, identifying 168 MTs in 37 species of ascidians, 4 of thaliaceans, and 3 of appendicularians, representing

planktonic and benthic species, as well as solitary and colonial forms. We have also functionally characterized the metalbinding properties of eight tunicate MTs, which were selected on the basis of their taxon representativeness, sequence composition, degree of multiplicity, or modular organization. Comparative analyses of tunicate MTs with those of cephalochordates and vertebrates have exposed a high degree of structural diversity between lineages, which would not have been accompanied, however, by differences in their metal preferences. This diversity seems the result of numerous and intricate evolutionary events, and therefore, chordate MTs would represent one of the most complex and enigmatic examples of protein evolution.

#### Results

The biological interest for tunicates in the last decades has given rise to many genomic and transcriptomic resources, facilitating the in silico identification of virtually any gene in a wide set of species. This advantageous situation allowed us to perform an extensive survey of the MT system in tunicate databases.

## MTs in Ascidians

Ascidians, commonly known as sea squirts, are the most studied group of tunicates. Adults are sessile, either solitary or colonial, usually found in shallow water worldwide. We surveyed ascidian databases and identified 145 MT sequences from 37 species distributed among Aplousobranchia (three species), Phlebobranchia (eight species), and Stolidobranchia (3 molgulidae + 5 pyuridae + 18 styelidae species) orders (fig.)1; see supplementary table S1, Supplementary Material online, for the species list). Sequence comparison showed that most ascidian MTs are 38-45 amino acids long with 12 cysteines (31–27%) organized in a single functional domain. In this domain. the C-motifs were distributed as  $[CxC]x_5[CxC]x_2Cx_2Cx_2[CxC]x_4[CxC]x_{3-6}CC$ , which would represent the prototypical organization of the monomodular ascidian (supplementary MTs fig. S1, Supplementary Material online, and table 1).

Twenty-seven out of the 37 analyzed species showed multiple MT genes: seven species with two sequences, six with three, two with four, three with six, two with seven, four with eight, one with nine, and two species with up to ten sequences. Despite in some cases, due to the nature of the data source—mainly sequence read archive (SRA) projects–, allelic variants of a particular gene could not be discriminated from gene duplicates, overestimating thereby the degree of MT multiplicity in our catalog, our results suggested that, overall, the MT multiplicity in ascidians is pervasive, although a definitive evaluation of the degree of multiplicity must await more complete and deeper genomic databases.

Interestingly, there were some exceptions to the prototypical organization, ranging from MTs that have lost or gained some cysteines or small protein fragments (supplementary fig. S1, Supplementary Material online) to multimodular forms containing tandem repeats of the prototypical domain (supplementary figs. S2 and S3, Supplementary Material online). We were able to reconstruct 12 multimodular MTs (five partial



Fig. 1. Phylogenetic relationships in the *Chordata* phylum. Regarding tunicates, traditional classifications divided this subphylum in three classes, Appendicularia (green background), Ascidiacea (yellow background), and Thaliacea (blue background), but recent analyses propose Thaliacea species (salps, doliolids, and pyrosomes) are nested within the Ascidiacea class, closer to the Aplousobranchia and Phlebobranchia orders than to the Stolidobranchia order (Delsuc et al. 2018; Kocot et al. 2018). A schematic representation of the MTs of each taxonomic group, with a mono-, bi-, or multimodular organization with distinct domains (color coded) containing seven, nine, 11, or 12 cysteines, is showed at the right (see fig. 7 and text for additional details).

Table 1. C	<b>Cysteine Motifs</b>	n 11/12-Cys	Domains from	n Different Animal	Taxa.
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Taxon	11/12-Cys Domains	Cysteine Motifs	Metal lons
Ascidians/thaliacea	nsFull-length MT	[CxC]x <sub>5</sub> [CxC]x <sub>2</sub> Cx <sub>2</sub> Cx <sub>2</sub> [CxC]x <sub>4</sub> [CxC]x <sub>3-6</sub> CC	4 (this work)
Appendicularians	12C domain	Cx <sub>3</sub> Cx <sub>2</sub> Cx <sub>2</sub> Cx <sub>3</sub> Cx <sub>2</sub> [CxC]x <sub>2</sub> Cx <sub>4</sub> [CxC]xCC	4 (Calatayud, Garcia-Risco, Capdevila, et al. 2021)
Vertebrates	α-domain <sup>a</sup>	CCxCCx <sub>3</sub> Cx <sub>2</sub> Cx <sub>3</sub> [CxC]x <sub>4-12</sub> Cx <sub>1-3</sub> CC	4 (Stillman et al. 1987)
Cephalochordates	11C domain	[CxC]x <sub>5</sub> [CxC]x <sub>3-4</sub> [CxC]x <sub>3</sub> [CxC]x <sub>3</sub> [CxC]x <sub>2</sub> C	nd
Mollusks	α-domain	[CxC]x <sub>4-6</sub> [CxC]x <sub>3</sub> Cx <sub>4-5</sub> [CxC]x <sub>3</sub> [CxC]x <sub>3</sub> [CxC]x <sub>2</sub> C	4 (Digilio et al. 2009)
Echinoderms	α-domain	CxCCx5[CxC]x4CCx4CCx4CC	4 (Tomas et al. 2013)
Insects	MTnB/C/D	$Cx_2Cx_3[CxC]x_4Cx_3[CxC]x_3[CxC]x_C]x_7CC$	4 (Egli et al. 2006)

NOTE.-nd, not determined.

<sup>a</sup>Notice that although vertebrate, echinoderm, and mollusk MTs have α-domains, the origin and evolutionary relationship of these α-domains remains uncertain.

and seven full-length sequences), whose number of repeats ranged from two to nine (fig. 2). Noteworthy, all the species with multimodular MTs belonged to the styelidae family in the stolidobranchia order (fig. 1), suggesting that multimodular MTs arose by a lineage-specific evolutionary event.

# Metal-Binding Features of Mono-Modular Ascidian MTs

In order to analyze the metal-binding capacity and preference of the ascidian MTs, we selected three MTs of two species belonging to distinct orders within the Ascidiacea class: CroMT1 from *C. robusta* (phlebobranchia order), and HroMT1 and HroMT2 from *Halocynthia roretzi* (stolidobranchia order). These MTs were chosen because CroMT1 was the only MT in *C. robusta*, whereas HroMT1 and HroMT2 represented duplicate forms in *H. roretzi*. These MTs were also selected because of their *K/N* ratios, which in the CroMT1 was 3:5, opposite of that in HroMT1 and HroMT2 sequences, 6:3 and 8:2, respectively. HroMT2 showed indeed one of the highest *K/N* ratios among tunicate MTs (supplementary table S1, Supplementary Material online). The *K/N* ratio seems important for metal preference of gastropod MTs (Perez-Rafael

1 MT	12C-R1	12C-R2	]						
5 T <sup>1 MT</sup>	12C-R1	12C-R2	12C-R3	12C-R4	]				
2 MTs	12C-R1	12C-R <sub>2</sub>	12C-R <sub>3</sub>	12C-R4	12C-Rs	12C-R6			
Y 3 MTs	12C-R1	12C-R <sub>2</sub>	12C-R <sub>3</sub>	12C-R4	12C-Rs	12C-R6	12C-R7	]	
2 MTs	12C-R1	12C-R2	12C-R <sub>3</sub>	12C-R4	12C-R5	12C-R6	12C-R7	12C-R8	
E 3 MTs	12C-R1	12C-R2	12C-R <sub>3</sub>	12C-R4	12C-Rs	12C-R6	12C-R7	12C-R8	12C-R9

**Fig. 2.** Schematic representation of multimodular Stelydae MTs. Internal duplications of a 12-Cys domain (grey box) generated multimodular MTs with variable number of repeats (R), from 2 to 9. The number of MTs with different number of repeats ( $R_1-R_9$ ) is indicated (DgrMT7 = 2 repeats; EtiMT8part  $\geq$ 4 repeats; ScaMT6part and SsoMT8part  $\geq$ 6 repeats; PmiMT10part, BleMT1, and BleMT2  $\geq$ 7 repeats; PauMT10part and PspMT7  $\geq$ 8 repeats; BscMT1, PmamiMT3, and PpoMT7 = 9 repeats) (see supplementary fig. S2, Supplementary Material online, for further details).

et al. 2014; Pedrini-Martha et al. 2020), and the study of the metal selectivity of the CroMT1, HroMT1, and HroMT2 would allow us to experimentally evaluate the K/N rule in the ascidian system.

We characterized the formation of metal-MT complexes of CroMT1, HroMT1, and HroMT2 proteins heterologously expressed in Escherichia coli cultures supplemented with Cu, Cd, or Zn salts by inductively coupled plasma atomic emission spectroscopy (ICP-AES) and electrospray ionization mass spectrometry (ESI-MS). ICP-AES was used for protein quantification and metal-toprotein stoichiometry determination through the measurement of element composition of the samples (S, Zn, Cd, and Cu) (Bongers et al. 1988), and ESI-MS allowed us to determine the molecular mass of the species formed, that is, the speciation of the samples (Capdevila et al. 2012). Single well-folded Cd<sub>4</sub>-MT species were recovered from CroMT1, HroMT1, and HroMT2 Cd-productions (fig. 3A-C), and a major  $Zn_4$ -MT species were found in their recombinant Zn productions (supplementary fig. S4A, C, and E, Supplementary Material online), which contrasted with the mixtures of homometallic Cu-MT species purified from Cu-supplemented E. coli cultures (supplementary fig. S4B, D, and F, Supplementary Material online). It is also noteworthy the resemblance of the CD spectra of the Cd-MT complexes of all ascidian MTs, denoting structurally equivalent metal clusters for these proteins (fig. 4). The exemplary exciton coupling centered at  $\sim$ 250 nm exhibited in all the CD envelopes confirms high robustness and compactness of the  $Cd_4S(Cys)_{12}$  clusters and, therefore, high Cd specificity. Altogether, these results showed that Zn<sub>4</sub>- or Cd<sub>4</sub>-MT complexes were energetically the most favorable forms of these proteins, suggesting that their function would be related to binding divalent metal ions, either as Zn- or Cd-thioneins [8]. Genuine Zn-thioneins, however, render heterometallic Cu, Zn-MT complexes when synthesized under Cu surplus, meaning that the ascidian MTs deviated toward a more specific Cd-thionein behavior (Palacios, Atrian, et al. 2011). Regarding the K/Nrule used to predict the metal-binding preference in gastropod MTs, our results demonstrated that this rule did not apply to tunicate MTs, since the three of them displayed contrasted K/N ratios and their metal preferences were exactly the same.

# Metal-Binding Features of Multimodular Ascidian MTs

In order to determine the metal-binding features of multimodular MTs, we selected BscMT1 (ID: ATSW01006707.1; chromosome 6) of Botryllus schlosseri (stolidobranchia order) because this MT was not only identified in an accurately assembled genome (Voskoboynik et al. 2013), but also because it was supported by three EST sequences (supplementary table S1, Supplementary Material online). BscMT1 was a multimodular MT of 365 amino acids with 105 cysteines (29%) and a K/N ratio of 1:1 (31K vs. 31N) (supplementary fig. S2 and table S1, Supplementary Material online). BscMT1 was one of the longest MT described so far, and a detailed analysis of its sequence revealed that it was made of nine tandem repeats  $(R_1 - R_9$ , the last one only partially complete) of a 12-Cys domain, each one resembling a prototypical ascidian form (supplementary fig. S3, Supplementary Material online).

The metal-BscMT1 complexes purified from the heterologous expression in E. coli cultures supplemented with Cu, Cd, or Zn salts were characterized by the IPC-AES and ESI-MS. The concentrations of metal-BscMT1 productions, despite the several attempts, were always low, probably due to the high molecular weight of BscMT1 and its structural complexity made of repeated domains. Only the samples recovered from the Zn(II)-enriched culture media rendered informative data through these techniques. The IPC-AES and ESI-MS analyses showed that this preparation contained Zn<sub>36</sub>-MT complexes (fig. 3D). The binding of 36 metal ions agreed with its multimodular organization made of nine repeated domains, each one similar to a prototypical  $M_4$ -MT: 4 metal ions×9 domains = 36 metal ions. To further verify the functional correspondence between one of the 12-Cys tandem repeats of BscMT1 and the mono-modular ascidian MTs, we analyzed the ability of the  $R_4$  domain ( $R_4$ -BscMT1) to form metal complexes when it was expressed alone. The election of the  $R_4$  domain was due to the fact that it was highly similar to the prototypical ascidian MTs, including some additional amino acids after the carboxyl-terminal CC doublet (supplementary fig. S3, Supplementary Material online). We heterologously expressed the R<sub>4</sub> domain in E. coli grown in media supplemented with Cu, Cd, or Zn salts. The ICP-AES and ESI-MS analyses of the recovered samples confirmed the ability of this fragment to autonomously form metal complexes by itself,



**FIG. 3.** Deconvoluted ESI-MS spectra of the Cd(II)-productions of ascidian CroMT1 (A), HroMT1 (B), HroMT2 (C), and +14 charge state ESI-MS spectra of the Zn(II)-production of BscMT1 (D), as well as the deconvoluted ESI-MS spectra of the Cd(II)-production of its fragment R<sub>4</sub> BscMT1 (E). Notice that BscMT1 is capable to bind up to 36 divalent ions, which nicely match with a multimodular organization made of nine repeated domains (12C)<sub>9</sub>, each one binding four divalent ions alike the mono-modular (12C) MTs (A–C) or the R<sub>4</sub> (12C) domain (E).



**FIG. 4.** Circular dichroism spectra of the Cd(II)-productions of CroMT1, HroMT1, HroMT2, BscMT1, and R<sub>4</sub> BscMT1, all exhibiting very similar CD signals. The exciton coupling centered at  $\sim$ 250 nm confirms high robustness and compactness of the clusters formed.

and revealed that the biochemical features of  $R_4$  domain were highly similar to the mono-modular ascidian forms (compare fig. 3A-C and E). Thus, this domain yielded major  $M_4-R_4$ complexes with Zn(II) and Cd(II), being unique species only in the latter, whereas rendered a homometallic mixture of species in the presence of copper, as the ascidian MTs discussed above (supplementary fig. S4G and H, Supplementary Material online). Based on this  $R_4$  fragment as a representation of the whole protein, the multidomain BscMT1 would be a Cd-thionein capable to bind up to 36 divalent ions, and its stoichiometry—that is,  $M_{36}$ -BscMT1—would nicely match with what would be expected from the repetition of nine domains, that is,  $M_4$ - $R_1$  to  $M_4$ - $R_9$ . These results supported a direct relationship between the binding capacity and the number of the repeated domains in multimodular MTs.

#### MTs in Thaliaceans

We analyzed the thaliacean MTs by surveying the databases of the five available species. We identified 14 MTs from four thaliacean species, three from Salpida (Iasis cylindrica, Salpa fusiformis, and Salpa thompsoni), and one from Pyrosomata (Pyrosomella verticillata) orders. Sequence comparison showed that thaliacean MTs were 36-41 amino acids long, with 12 cysteines (33-29%) organized in a single functional domain with the C-motifs distributed as  $[CxC]x_5[CxC]x_2Cx_2Cx_2[CxC]x_4[CxC]x_{3-4}CC$  (noteworthy, we did not find multimodular MTs in thaliacean databases) (table 1). This motif arrangement was identical to that of the prototypical ascidian MTs, denoting a common origin of that thaliacean and ascidian MTs, and supporting the recently suggested phylogenetic relationship of these classes (Delsuc et al. 2018; Kocot et al. 2018) (fig. 1).

Importantly, MT multiplicity was found in the four thaliacean species although as in ascidians, it was challenging to determine whether the multiple sequences represented different genes or allelic variants. In S. thompsoni, for instance, we identified six MT sequences, five full-length (SthMT1, MT2, MT3, MT5, and MT6), and one partial (SthMT4), which coded for proteins 48% to 95% identical at amino acid level (supplementary table S2, Supplementary Material online). The highest identities were between SthMT1 and SthMT5 (88%) and SthMT2 and SthMT6 (95%) sequences, and therefore, these pairs were the most plausible cases of allelic variants. To investigate this possibility, we analyzed the conservation of 5' and 3' flanking regions, as well as of intron sequences in PCR-amplified genomic regions from two S. thompsoni specimens. Nucleotide identity (excluding gaps) in SthMT1-SthMT5 and SthMT2-SthMT6 alignments was 91-100%, 87-82% and 83-86% at 5' regions, introns and 3' regions, respectively (supplementary table S3, Supplementary Material online). These results would be compatible with that SthMT5 and SthMT6 were allelic variants of SthMT1 and SthMT2, respectively, although the possibility they may be recently duplicated genes cannot be fully discarded.

#### Metal-Binding Features of Thaliacean MTs

We analyzed the metal-binding capacity and metal preference of the thaliacean MTs, and compared them with those of ascidian MTs in order to investigate whether the differences in lifestyles-planktonic versus benthic-might have been key for MT evolution. We characterized the metal-MT complexes rendered for the four MTs from S. thompsoni, SthMT1 to SthMT4, heterologously expressed in Zn-, Cd-, or Cu-supplemented E. coli cultures. The clear Cd-thionein character of S. thompsoni MTs was reflected, once again, by the ICP-AES and ESI-MS analyses of the recovered samples. SthMTs yielded single Cd<sub>4</sub>-MT species under Cd(II) surplus (fig. 5), as well as major  $M_4$ -MT complexes with Zn(II) (supplementary fig. S5A-D, Supplementary Material online). A mixture of species (for SthMT1 and SthMT3) or not detection of the metallated species (for SthMT2 and StMT4) were obtained in the presence of copper (supplementary fig. S5E and F, Supplementary Material online), evidencing that these proteins struggle to build metal clusters with Cu. Our results

demonstrated that the four SthMTs were functionally very similar among them (compare panels A–D in fig. 5), as well as to the mono-modular ascidian MT (compare fig. 3 and fig. 5). The fact that thaliacean (SthMTs) and ascidian (CroMT1, HroMT1, HroMT2, and BscMT1) MTs exhibited a similar Cd-thionein character strongly supported that the prototypical ascidian/thaliacean MT (and most likely the ancestral tunicate MT, see below) was a mono-modular protein with a 12C domain capable to bind four metal ions with preference for Cd.

#### MTs in Appendicularians

We investigated the MTs in the Appendicularia (a.k.a. Larvacea) class. In a previous work, we had characterized the OdiMT1 and OdiMT2 genes (formerly OdMT1 and OdMT2) of O. dioica (Calatayud et al. 2018). We showed that both genes encoded MTs very different from all MTs described so far because their cysteines motifs were arranged in a novel pattern forming a "repeat unit" (RU) made of two C7 subunits (C7a and C7b) plus a carboxyl-terminal tail with five cysteines (C5) (Calatayud et al. 2018) (supplementary fig. S6A, Supplementary Material online). We concluded that OdiMT1 was made of a single RU, whereas OdiMT2 was a multimodular protein made of six direct tandem RU (Calatayud et al. 2018). We wondered whether these unconventional MTs were specific of O. dioica-gene and protein sequences are known to have considerably diverged during O. dioica evolution (reviewed in Ferrández-Roldán et al. [2019])—or they were widespread in the appendicularian lineage. We took advantage of recent sequence projects of several appendicularian species (Naville et al. 2019) and identified seven new MT sequences in two additional Oikopleura species: O. albicans (three sequences) and O. vanhoeffeni (four sequences) (supplementary table S1, Supplementary Material online). We did not identify any putative MT in databases of other oikopleurids such as O. longicauda, Bathochordaeus sp., and Mesochordaeus ervthrocephalus, nor in the fritillarid species Fritillaria borealis, although any conclusion about the loss of the MTs in these lineages must await deeper sequence projects.

Regarding Oikopleura MTs, although most of the sequences were partial, they were clearly similar to O. dioica MTs. Sequence analyses showed that these new MTs were multimodular proteins made of repeats of a conserved 12-Cys domain with C-motifs distributed as CX<sub>3</sub>Cx<sub>2</sub>Cx<sub>2</sub>Cx<sub>3</sub>Cx<sub>2</sub>[CxC]x<sub>2</sub>Cx<sub>4</sub>[CxC]xCC (table 1 and supplementary fig. S6B, Supplementary Material online). This 12-Cys domain corresponded to the previously described C7b+C5 subunits (Calatayud et al. 2018). OdiMT1 consist, therefore, of two 12-Cys domains, one full-length (12C = C7b + C5), and one 12-Cys domain that has been trimmed (t-12C, formerly known as C7a) and lacks the five last cysteines (C5) (supplementary fig. S6A, Supplementary Material online and fig. 7). Interestingly, the trimmed domain when expressed alone did not efficiently bind divalent metals by itself, suggesting that it has lost its capacity to independently work (Calatayud, Garcia-Risco, Capdevila, et al. 2021). OdiMT2 would be a multimodular MT made of twelve 12-Cys domains, whose



FIG. 5. Deconvoluted ESI-MS of the Cd(II)-production of S. thompsoni SthMT1 (A), SthMT2 (B), SthMT3 (C), and to SthMT4 (D). Thaliacean MTs are mono-modular proteins made of a single 12C domain capable to bind four divalent ions, identically to ascidian MTs.

odd domains would have been also trimmed as t-12C. The identification of this conserved 12-Cys domain in all *Oikopleura* MTs suggested that this domain appeared in the ancestor of the *Oikopleura* genus, and that it was duplicated generating multimodular MTs independently in the diverse appendicularian species (supplementary fig. S6B, Supplementary Material online).

#### MTs in Other Chordates

To provide a broad evolutionary perspective to our analyses we compared the tunicate MTs with those from the other chordate subphyla: cephalochordates and vertebrates.

In cephalochordates, MTs had been characterized in two amphioxus species, Branchiostoma floridae and Branchiostoma lanceolatum (Guirola et al. 2012). We extended the surveys of MTs to two additional amphioxus species, Branchiostoma belcheri (BbeMT1 and BbeMT2) and Branchiostoma japonicum (BjaMT1 and BjaMT2), and to Asymmetron lucayanum (AluMT1 and AluMT2), a cephalochordate distantly related to the other Branchiostoma species (Yue et al. 2014). We identified two MTs, MT1 and MT2, in all the cephalochordate species. B. lanceolatum MT2 showed two alternatively spliced forms, MT2-L (for the long form, with 67-70 amino acids) and MT2-S (for the short form, with 46-47 amino acids) (supplementary fig. S7, Supplementary Material online). Splice variants were also identified in B. floridae, B. japonicum, and A. lucayanum cDNA databases (i.e., BflMT2L and S, BjaMT2L and S, and AluMT2L and S), and alternative splicing was predicted

from genomic sequence in *B. belcheri* species (BbeMT2L and S).

Sequence comparisons revealed that cephalochordate MT2s were multimodular proteins made of two (MT2S) or three (MT2L) 9-Cys domains (CCx<sub>2</sub>Cx<sub>2</sub>Cx<sub>3</sub>Cx[CxC]xCC) depending on an alternative splicing on exon 2 (supplementary figs. S7 and S8B, Supplementary Material online). In contrast, MT1s were bimodular proteins with two different domains: a 11-Cys, N-terminal domain with the C-motifs distributed as  $[CxC]x_5[CxC]x_{3-4}[CxC]x_3[CxC]x_3[CxC]x_2C$  (table 1 and supplementary fig. S8A, Supplementary Material online) linked by 9-11 residues to a domain similar to the 9-Cys domain of MT2 (we named this domain 9-Cys<sub>Like</sub> because an Ile substitutes the fourth Cys: CCx<sub>2</sub>Cx<sub>2</sub>Ix<sub>3</sub>Cx[CxC]xCC), followed by a carboxyl-terminal tail of 16-17 amino acids devoid of Cys residues. Interestingly, MT2 9-Cys and the MT1 9-Cyslike domains showed a clear sequence similarity (supplementary fig. S8C, Supplementary Material online), which suggested that cephalochordate MT2s might have arisen from the duplication of an ancestral MT1 that lost the 11-Cys domain but amplified its 9-Cys domain.

In vertebrates, MTs had been classified in four different MT types: MT1, MT2, MT3, and MT4. Vertebrate MTs had been characterized in many species with significant differences of multiplicity in a species-dependent manner (Serén et al. 2014). Phylogenetic and synteny analyses had suggested the existence of mammalian MT1s and MT2s, amniote MT4s, tetrapod MT3s, and fish MTs (fig. 1) (Serén et al. 2014). The appearance of these four types would not be related with the two rounds

of whole-genome duplication events that occurred during early vertebrate evolution since they are not localized in paralogous chromosomes but are located in tandem on a same chromosome (Serén et al. 2014). At protein level, all vertebrate MTs (even an MT we identified in the early divergent group of hagfish Eptatretus burgeri), showed a conserved bimodular organization made of a 9-Cys N-terminal domain (the socalled  $\beta$ -domain: [CxC]x<sub>5</sub>[CxC]x<sub>3</sub>[CxC]x<sub>2</sub>[CxC]x<sub>2</sub>Cx<sub>3</sub>) linked to 11-Cys domain (the so-called  $\alpha$ -domain: а  $CCxCCx_3Cx_2Cx_3[CxC]x_6CxCC)$  at the carboxyl-terminal end (supplementary table S1 and fig. S9, Supplementary Material online). The arrangement of the C-motifs in the  $\alpha$  and  $\beta$ domains did not show any obvious resemblance between them that might suggest of a common ancestry of these domains, though vertebrate  $\beta$ -domain appeared somewhat similar to cephalochordate and tunicate 11/12-Cys domains.

## Discussion

#### Origin and Convergent Evolution of Tunicate MTs

Ascidian and thaliacean MTs share the same arrangement of their C-motifs (table 1) and are clearly similar in their amino acid sequence, also in the noncysteine residues: ascidian MTs are at least  $\approx$ 40% identical in their noncysteine residues to any of the thaliacean MTs, and  $\approx$ 60% identical when considering the full sequence. In contrast, their similarity with appendicularian MTs is negligible at the arrangement of the C-motifs (table 1) and in the composition of the noncysteine residues. For instance, OdiMT1 only is, at most, 13% identical to ascidian (e.g., CroMT1 and HroMT1) or thaliacean (e.g., SthMT1) MTs in noncysteine residues (notice that 25-30% sequence identity is generally taken as the minimal threshold for presumption of homology; Rost 1999). Therefore, although it cannot be ruled out that appendicularian MTs derive from ascidian and thaliacean MTs that have diverged too much for orthology to be recognized, their sequence and the C-motifs are so divergent that they seem to have had independent evolutionary origins. This possibility agrees with the idea that de novo emergence of MTs might be more habitual than previously thought because the only requirement for a peptide to function as a metal ion chelator would be a high content of coordinating residues (i.e., cysteines) and a relative small length that would favor its proper folding (Capdevila and Atrian 2011; Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021). The independent emergence of MTs in distant animal groups have been, indeed, implicitly suggested in diverse evolutionary studies (Capdevila and Atrian 2011; Blindauer 2014; Isani and Carpene 2014; Ziller and Fraissinet-Tachet 2018), and we have recently showed de novo evolution of MT domains within the Mollusca phylum (Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021).

Intriguingly, ascidian/thaliacean MTs and appendicularian MTs seem to have converged in the number of cysteines— 12—of their basic functional organizations: the prototypical 12-Cys MTs of ascidians and thaliaceans, and the repeated 12-Cys domains of appendicularian MTs. Functional domains with 11/12 cysteines organized in different patterns of Cmotifs but converging to similar 3D architectures (Beil et al. 2019) appear a recurrent evolutionary solution in MTs of phylogenetically distant animal clades, from vertebrates and tunicates to mollusks and insects (table 1). 3D structural constraints to efficiently coordinate four metal ions through the formation of metal-thiolate bonds might have led to the convergent evolution of 11/12-Cys domains in MTs of different animal phyla (Stillman et al. 1987; Egli et al. 2006; Digilio et al. 2009; Tomas et al. 2013; Calatayud, Garcia-Risco, Capdevila, et al. 2021; and this work).

#### Evolution of the Metal-Binding Preference

Ascidian/thaliacean MTs and appendicularian MTs also share the Cd-thionein character. The evolution of Cd-MTs seems, indeed, a common adaptive event in marine animals, including mollusk (Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021), crustacean (Narula et al. 1995; Valls et al. 2001; Munoz et al. 2002), echinoderm (Riek et al. 1999), and chordate species (Guirola et al. 2012; Calatayud et al. 2018). The preference of tunicate MTs for Cd further support the idea that ancestral MTs arose and evolved probably driven by the Cd(II) concentrations in the seawater because Cd is highly toxic by interfering in Zn-dependent cellular processes (Dallinger et al. 2020; Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021). In addition to sporadic episodes of increases in Cd emissions throughout geological eras (from Paleozoic to Mesozoic and Cenozoic; Dallinger et al. [2020] and references therein), Cd is frequently found with Zn in ore deposits of the earth crust. The natural Cd(II) concentrations are, therefore, higher where seawater comes into contact with the continental earth crust (pprox0.8 nmol/kg) than in surface pelagic waters ( $\approx$ 0.002 nmol/kg) (Bruland 1980). Cd(II) concentrations are also higher in eutrophic coastal regions of the littoral and its close neritic zone ( $\approx$ 0.16 nmol/kg) (Bruland 1980), which are usual habitats for tunicates. It is noteworthy that all tunicate MTs analyzed in this work show a similar sequence and metal preference, either they are from benthic ascidians or from planktonic thaliaceans, or from solitary (C. intestinalis and H. roretzi) or colonial species (B. schlosseri). This similarity suggests that the different lifestyles of tunicates might have not been a significant factor in the evolution of metal-binding preference.

#### Evolution of MT Multiplicity and Multimodular MTs

Increasing the metal-binding capacity enhance the physiological capabilities of the organisms to adapt to diverse conditions of metal bioavailability and other environmental stress situations (Jenny et al. 2016; Baumann et al. 2017). In a simplified view, such increments might be attained through two strategies: increasing the number of *MT* genes (i.e., augmenting MT multiplicity) (Maroni et al. 1987; Adamo et al. 2012; Luo et al. 2020) or increasing the number of metal-binding domains in a given MT (i.e., generating multimodular MTs) (Jenny et al. 2016; Baumann et al. 2017). Tunicates have used both strategies.

#### Evolution of MT Multiplicity

Multiplicity in the MT system has been associated to neofunctionalization or subfunctionalization processes, in which different paralogs have distinct metal-binding preferences and/or expression patterns. For instance, in mollusks, several MTs can be found in a given species, each one with distinct metal selectivity-that is, Cd-MT, Cd/Cu-MT, and Cu-MTand differential transcription patterns (Palacios, Perez-Rafael, et al. 2014; Dvorak et al. 2018; Dallinger et al. 2020). Similarly, in the mammalian system, ubiquitous metal-induced forms MT1 and MT2 have preference for Zn(II), whereas restrictedly expressed MT3 and MT4 have intermediate Zn/Cu- and Cuthionein characters (Capdevila and Atrian 2011; Vasak and Meloni 2011; Artells et al. 2013). MT multiplicity has been observed in 36 tunicate species, patchily distributed among the three tunicate classes. However, in contrast with the observed in the MT system of other animal groups, the analyzed tunicate MT paralogs do not seem to have essentially diverged in their metal-binding selectivity. The two paralogs of the ascidian H. roretzi (HroMT1 and HroMT2), the four paralogs of the thaliacean S. thompsoni (SthMT1 to SthMT4), and the two paralogs of the appendicularian O. dioica (OdiMT1 and OdiMT2; Calatayud et al. 2018) have a conserved Cd selectivity. These results suggest that tunicate paralogs have undergone a subfunctionalization process partitioning the expression of the predecessor, rather than a neofunctionalization event acquiring new metal preferences. Future detailed spatiotemporal analyses of the transcription patterns of each paralog will provide a definitive answer to this question, and will allow us to understand the ecophysiological needs that led the evolution of multiple MTs in these species.

#### **Evolution of Multimodular MTs**

Evolution of multimodular MTs seems a recurrent solution for creating large MTs with high cysteine content and a high capacity of metal binding (Niederwanger et al. 2017; Palacios et al. 2017; Calatayud et al. 2018; and this work). Multimodular MTs have been found in very diverse organisms, from fungi (Palacios, Espart, et al. 2014; Iturbe-Espinoza et al. 2016) and mollusks (Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021; and references therein), to chordates (Guirola et al. 2012; Calatayud et al. 2018). Here, we have identified multimodular MTs in 20 species of ascidian and appendicularian classes (thaliaceans appear devoid of multimodular MTs). These multimodular MTs are made of tandem repeats of a prototypical organization within each tunicate class— $[CxC]x_5[CxC]x_2Cx_2Cx_2[CxC]x_4[CxC]x_{3-6}CC$ for ascidian and CX<sub>3</sub>Cx<sub>2</sub>Cx<sub>2</sub>Cx<sub>3</sub>Cx<sub>2</sub>[CxC]x<sub>2</sub>Cx<sub>4</sub>[CxC]xCC for appendicularian MTs-, implying independent evolutionary origins for ascidian and appendicularian multimodular MTs. In ascidians, the multimodular MTs arose during the evolution of the Styelidae family in the Stolidobranchia order, after its split from the Pyuridae family, 277 Ma (Delsuc et al. 2018). In appendicularians, multimodular MTs arose after its divergence from the other tunicate classes, 447 Ma (Delsuc et al. 2018). The adaptive causes that favored the evolution of large MT in some benthic ascidian species but not in others, or in some planktonic tunicates such as appendicularians but not in thaliaceans, are still an unsolved question.

#### The Enigmatic Origin of the MTs in Chordates

The extensive catalog of tunicate MTs collected in this work together with the analysis of cephalochordate and vertebrate MTs has allowed us to investigate the origin and evolution of the MTs in the chordate phylum. This has been a particularly difficult challenge because when comparing distant taxonomic groups (e.g., at phylum, subphylum, or class level), one has to be cautious with the MT sequence alignments. The general cysteine richness of the MTs distorts the comparisons by forcing the alignment of Cys positions and inserting many gaps in the aligned sequences. This distortion results in a false impression of similarity between nonhomologous MT sequences, and if the alignments are used for phylogenetic reconstructions, they can lead to wrong evolutionary inferences. To circumvent this drawback, we focused our analyses on the patterns of the cysteine motifs (CxC, CC, and CCC) in the MT domains that, as it has been proved for other molecular markers (Rokas and Holland 2000), might have enormous potential as a tool for phylogenetic inferences.

Regarding the ancestral chordate MT, it is generally accepted that due to their substantial genomic stasis (Cañestro and Albalat 2012; Somorjai et al. 2018; Coppola et al. 2019), cephalochordate genes and genomes are more similar to those of the chordate ancestor than those of tunicates and vertebrates (Louis et al. 2012). It can be argued, therefore, that the MT of the chordate ancestor was a bimodular protein with an amino-terminal 11/12-Cys domain and a carboxyl-terminal 9-Cys domain (fig. 6), resembling the organization of the current cephalochordate MT1s. The similarity of cephalochordate and vertebrate domains (fig. 7) supports the bimodular organization of the ancestral chordate MT. In addition, the fact that cephalochordate MT1s are also similar to the  $\alpha$  and  $\beta$ 1 domains of mollusks (Calatayud, Garcia-Risco, Pedrini-Martha, et al. 2021) would push back in evolutionary time this MT organization to the origins of the bilaterian animals.

The evolutionary scenario of a chordate MT ancestor similar to the current cephalochordate MT1 implies a number of evolutionary events independently shaping the MTs in each chordate subphylum: (I) In cephalochordates, an ancient tandem duplication of the ancestral MT followed by the loss of the 11-Cys domain and the amplification of the 9-Cys domain in one of the copies would have led the MT2 forms of the subphylum (fig. 6); (II) In vertebrates, the ancestral MT would have readjusted its Cys motifs, leading to the current aminoterminal  $\beta$ - and the carboxyl-terminal  $\alpha$ -domains (fig. 6). Successive lineage-specific tandem duplications would have led to a variable degree of the MT multiplicity in the different vertebrate species (Serén et al. 2014); (III) In tunicates, the ancestral MT would have lost the 9-Cys domain in the ascidian/thaliacean lineage. This mono-modular 12-Cys MT was patchily duplicated, giving rise to a variable number of MTs in different ascidian/thaliacean species, or internally repeated in the multimodular MTs of styelidae species (fig. 6); (IV) Finally, in the fast-evolving appendicularians, the ancestral chordate MT would have been lost, and a new MT with a different pattern of Cys motifs seems to have evolved de novo (fig. 6).

## MBE



**Fig. 6.** Reconstruction of MT evolution in the *Chordata* phyla. The most parsimonious scenario would be that the ancestral chordate MT was a bimodular MT made of an N-terminal 11/12-Cys domain (in blue) and a C-terminal 9-Cys domain (in red), similar to the current cephalochordate MT1. In cephalochordates, this ancestral form was tandem duplicated. One copy lost the 11/12-Cys domain and expanded the 9-Cys domain, yielding the current bi/trimodular MT2 form. In vertebrates, the number of cysteines in each domain was readjusted to the current 9 Cys of the N-terminal  $\beta$ -domains, and 11 Cys of the C-terminal  $\alpha$  domain. Successive lineage-specific tandem duplications of this primeval vertebrate MT led to different MT types (MT1, MT2, MT3, and MT4) with a variable degree of multiplicity in different species. In tunicates, the ancestral form lost the C-terminal 9-Cys domain in ascidian/thaliacean lineages, leading a mono-modular. This MT was frequently duplicated generating a high degree of MT multiplicity in many ascidian and thaliacean species. In contrast, internal duplications led to the emergence of multimodular MTs in the stelydae lineage. Finally, in the appendicularian clade, the ancestral MT appears to have been lost, and a new MT would have emerged de novo by the duplication of a 12-Cys domain (in green). During *O. dioica* evolution, the new bimodular MT lost five Cys in the N-terminal domain, yielding a 7-Cys/12-Cys arrangement (as it is found in current OdiMT1). Next, this 7-Cys/12-Cys MT was internal duplicated generating multimodular MTs (as it is OdiMT2).

The primeval appendicularian MT would have been a bimodular form made of two 12-Cys domains, apparently unrelated with the other 12-Cys domains of chordates. This ancestral appendicularian MT lost five Cys in the N-terminal domain in the *O. dioica* lineage (but remained intact in other *Oikopleura* lineages), yielding the t-12C + 12C organization of the current OdiMT1. This MT was later duplicated, and internal duplications in one of the copies of a repeat unit (RU) made of the t-12C + 12C domains gave rise to the large multimodular OdiMT2 (Calatayud et al. 2018).

In summary, our analyses have shown: 1) de novo emergence of MT genes and domains during chordate diversification; 2) convergent evolution to 12-Cys domains in chordate MTs, but also in other animals, probably favored by essential constraints of the metal coordination; 3) convergent evolution in the metal preference for Cd ions, probably triggered by an ancestral requirement for cadmium-selective forms in many marine animals; 4) lineage-specific events of gene duplications yielding significant levels of MT multiplicity; and 5) independent amplifications of domains that generated multimodular MTs with high metal-binding capacities. All these results have led us to reconstruct an intricate evolutionary history for the chordate MTs, from a bimodular, cadmiumselective form in the last common ancestor, to a high structural and functional MT diversity currently found in the diverse species of the phylum. To discover the adaptive causes that shaped this history and the molecular mechanisms that made it possible will certainly be a fascinating challenge.

## **Materials and Methods**

#### Chordate MT Search in Databases

Tunicate and cephalochordate MT sequences were retrieved from public databases (NCBI database: https://www.ncbi.nlm. nih.gov/; and Octopus database: http://octopus.obs-vlfr.fr/ public/botryllus/blastbotryllus.php) by BlastP and TBlastN searches using known chordate MTs as queries: tunicate CroMT1 (ACN32211), HcuMTa (AY314949), OdiMT1 (AYN64372), and OdiMT2 (AYN64376); cephalochordate





Fig. 7. Comparison of the arrangement of Cys motifs in different MT domains. The arrangement of the Cys motifs in the 11-Cys amino-terminal domain of cephalochordate MT1s are somewhat similar to that in the vertebrate 9-Cys  $\beta$  domains and in the ascidian/thaliacean 12-Cys MTs (blue boxes), whereas the Cys motifs in the carboxyl-terminal domains of cephalochordate MT1s and MT2s (9C and 9C<sub>Like</sub> domains) are vaguely similar to those in vertebrate 11-Cys  $\alpha$  domains (pink boxes). In contrast, the arrangement of the Cys motifs in the appendicularian MT1 seems totally different (green boxes).

BfIMT1 (XP 035672826), BfIMT2 (BW841405), BlaMT1 (BL11229), and BlaMT2 (JT872034); and vertebrate HsaMT1A (NP\_005937), HsaMT2A (NP\_005944), HsaMT3 (NP 005945), and HsaMT4 (NP 116324). In addition, RNA-SRA for each tunicate species deposited in NCBI were BLAST searched using as queries MT sequences from the nearest phylogenetic species as well as from different species covering the main tunicate clades. Raw sequence data were retrieved and assembled using SeqMan 8.0.2 (Pro Assembler) software from the DNASTAR Lasergene package, and manually inspected in order to reconstruct new MT sequences. The MT nature of each new identified sequence was evaluated by BlastX searches against metazoan NCBI nonredundant protein sequence database. The amino acid sequences and the accession numbers of the retrieved MTs are provided in supplementary table S1, Supplementary Material online. Sequence alignments were generated with Aliview program (Larsson 2014) and reviewed manually.

#### Characterization of S. thompsoni Genes

Salpa thompsoni specimens were kindly provided by Nora-Charlotte Pauli from the Bettina Meyer's group at Alfred Wegener Institute for Polar and Marine Research (AWI). Genomic DNA was extracted using Quick-DNA/RNA Microprep Plus Kit Protocol (Zymo Research, CA) using 5 mg of tissue and following the manufacturer's instructions. The DNA concentration, purity, and integrity were checked using Tecan Infinite M200 (Tecan Group Ltd, Switzerland) measuring the absorbance at 260 and 280 nm.

Based on S. *thompsoni* genome and transcriptome projects (PRJNA318929 and GFCC00000000.1, respectively) and the raw data from S. *thompsoni* SRA projects, several pairs of primers were designed in order to PCR amplify the 5' and 3' flanking regions, as well as of intron sequences of the different SthMTs (supplementary table S4, Supplementary Material online).

For SthMT1 we used the primer pairs FwMT1/MT5 with RvMT1/MT2; for SthMT5, FwMT1/MT5 with RvMT5, and finally, for SthMT6 we used FwMT2/MT6 with RvMT6.

For each PCR reaction, 1 ng of genomic DNA was amplified using selected pairs of primers and the Phusion High-Fidelity DNA Polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA) in a final 25  $\mu$ l reaction. PCR conditions were 98 °C 30 s (s); 35 cycles of 98 °C 10 s, 50-54 °C 30 s (supplementary table S4, Supplementary Material online) and 72 °C 3 min; and 72 °C 10 min. PCR products were visualized in 0.8% agarose gels, isolated with the GelElute Plasmid Miniprep Kit (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and cloned with TOPO TA Cloning Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Plasmid DNA was purified from bacteria using the GeneElute Plasmid Miniprep Kit (Sigma-Aldrich), screened for insert presence by digestion with Fast Digest EcoRI (Invitrogen, Thermo Fisher Scientific, Waltham, MA), and seguenced at the Scientific and Technological Centers of the University of Barcelona using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an automatic sequencer (ABIPRISM 310, Applied Biosystems). Sequences analyzed Unipro were with UGENE software (Okonechnikov et al. 2012).

#### Production and Purification of Recombinant Metal-MT Complexes

Synthetic cDNAs codifying the selected MTs (i.e., CroMT1, HroMT1, HroMT2, BscMT1, R<sub>4</sub>-BscMT1, SthMT1, SthMT2, SthMT3, and SthMT4) were provided by Synbio Technologies (Monmouth Junction, NJ), cloned in the pGEX-4T-1 expression vector (GE Healthcare, Chicago, IL), and transformed in protease-deficient E. coli BL21 strain for heterologous expression. For heterologous protein production, 500 ml of Luria–Bertani (LB) medium with 100  $\mu$ g/ml ampicillin was inoculated with E. coli BL21 cells transformed with the corresponding recombinant plasmid. After overnight growth at 37°C/250 rpm, the cultures were used to inoculate 5 l of fresh LB-100  $\mu$ g/ml ampicillin medium. Gene expression was induced with 100  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) for 3 h. After the first 30 min of induction, cultures were supplemented with  $ZnCl_2$  (300  $\mu$ M), CdCl<sub>2</sub> (300  $\mu$ M), or CuSO<sub>4</sub> (500  $\mu$ M) in order to generate metal-MT complexes. Cells were harvested by centrifugation for 5 min at 9,100  $\times$  g (7,700 rpm), and bacterial pellets were suspended in 125 ml of ice-cold phosphate-buffered saline (PBS: 1.4 M NaCl, 27 mM KCl, 101 mM Na<sub>2</sub>HPO<sub>4</sub>, 18 mM KH<sub>2</sub>PO<sub>4</sub>, and 0.5% v/v  $\beta$ -mercaptoethanol). Resuspended cells were sonicated (Sonifier Ultrasonic Cell Disruptor) 8 min at voltage 6 with pulses of 0.6 s, and then centrifuged for 40 min at 17,200 × g (12,000 rpm) and 4 °C.

Soluble protein extracts containing GST-MT fusion proteins were incubated with glutathione sepharose beads (GE Healthcare) for 1 h at room temperature with gentle rotation. GST-MT fusion proteins bound to the sepharose beads were washed with 30 ml of cold 1xPBS bubbled with argon to prevent oxidation. After three washes, GST-MT fusion proteins were digested with thrombin (SERVA Electrophoresis GmbH Heidelberg, Germany, 25 U/l of culture) overnight at 17 °C, thus enabling separation of the metal-MT complexes from the GST that remained bound to the sepharose matrix. The eluted metal-MT complexes were concentrated with a 3 kDa Centripep Low Concentrator (Amicon, Merck-Millipore, Darmstadt, Germany), and fractionated on a Superdex-75 FPLC column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 7.0. The protein-containing fractions, identified by their absorbance at 254 nm, were pooled and stored at -80 °C until use.

#### Analysis of Metal-MT Complexes

Protein quantification and element composition of all the samples were achieved by ICP-AES measurements performed in a Optima 4300DV apparatus (Perkin-Elmer, MA) (S, 182.040 nm; Zn, 213.856 nm; Cd, 228.802 nm; Cu. 324.803 nm) under conventional conditions following an already stablished method (Bongers et al. 1988). Molecular weights were determined by ESI-MS, in a MicroTof-Q instrument (Bruker Daltonics Gmbh, Bremen, Germany) connected to a Series 1100 HPLC pump (Agilent Technologies) controlled by the Compass Software. The instrument was calibrated with ESI-L Low Concentration Turning Mix (Agilent Technologies, Santa Clara, CA). Metallated forms were detected under native conditions: 20 µl of sample injected through a PEEK tube at  $30-50 \,\mu l \,min^{-1}$  in a  $3.5-5.0 \,kV$ capillary-counter voltage, at 90-110 °C of desolvation temperature, and with dry gas at  $61 \text{ min}^{-1}$ . The spectra were recorded between a m/z range from 800 to 3,000. The liquid carrier was a 90:10 mixture of 15 mM ammonium acetate and acetonitrile at pH 7.0. All molecular masses were calculated according to the bibliography (Fabris et al. 1996).

## **Supplementary Material**

Supplementary data are available at *Molecular Biology and Evolution* online.

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## Data Availability

The data underlying this article are available in NCBI (https:// www.ncbi.nlm.nih.gov/) and Octopus (http://octopus.obsvlfr.fr/public/botryllus/blastbotryllus.php) databases. The amino acid sequences and the accession numbers of the MTs used in this work are available in supplementary table S1, Supplementary Material online.

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**Figure S1. Amino acid alignment of Ascidian MTs.** Conserved cysteines are highlighted in yellow. Configurations of cysteine motifs are showed below each alignment. Names of MT sequences from different species are as in Supplemental\_Table\_S1.pdf, and alphabetically ordered.



Figure S2. Amino acid alignment of multimodular Stelydae Ascidian MTs. Conserved cysteines are highlighted in yellow. Configurations of cysteine motifs are showed below each alignment. Names of MT sequences from different species are as in Supplemental\_Table\_S1.pdf.

#### FIGURE S3

DgrMT7_R1	MNP	СT	C A D D G P	СH	<mark>C</mark> D Q <mark>(</mark>	C T D <mark>(</mark>	<mark>c</mark> ss <mark>c</mark>	R	<b>C</b> SSAN	<mark>c</mark> ĸ	<mark>C</mark> A T S G K S	<mark>c c</mark>	ббдт	44
DgrMT7_R2	G E P	СT	CADSGP	C R	CAQ	<mark>с</mark> те <mark>(</mark>	<mark>c</mark> sr <mark>c</mark>	R	C S P A N	CQ	<mark>C</mark> S K S G R T	<mark>c c</mark>	v	41
EtiMT8_R1	MN P	CK	<mark>C</mark> A E T G S	C R	C D R <mark>(</mark>	CTD	c s q <mark>c</mark>	2 Q C	C N P D N	C K	<mark>C</mark> A T S G K G	<mark>c c</mark>		40
EtiMT8_R2	QGP	CK	C A E G G P	СН	CDQ	CTD	C S N C	K	C A P G N	CK	<mark>C</mark> A G S G K E	<mark>c c</mark>		40
EtiMT8_R3	PRP	CK	C A E G G P	Сн	CDE	CTD	C S R C	K	<b>C</b> A P A N (	CK	<mark>C</mark> A K S G K S	c c		40
EtiMT8_R4part	PEA	CK	<b>C</b> A											7
ScaMT6_R1part					- D K (		<b>C</b>		CNPEA	CK	<b>C</b> A - SG		GSAT	27
ScaMT6_R2	VRP		CKETGT	СН			C S N C	K	CNPEM	CK	<mark>С</mark> Т-ТС	cc	ANTT	41
ScaMI6_R3part	VGP	CN	CKETGT	СН		CTDO								20
PSpMT7_R1	MNP	C K	CAETGS								CATSGKG			40
PSpMT7_RZ	QGP	CK	CAEGGP	Сн		CTDC		K			CAGSGKE	00		40
PSpMT7_R3	PRP		CAEGGP	Сн							CAKSGKS			40
PSpMT7_R4	PEA		CAEGGP								CAKSGKG			40
PSPHI7_RS	PGP		CAEGGP											40
PSpMT7_R0	PEP		CAEGGP											40
PSPHI7_R/	PGP										CATSGRA			40
PSPHI/_Ro	GRP		ADTGS					R			CATAGRQ		RGKA	44
SSOMTO_RI	ACP		CALTGS								CAASGRG			40
SSOMIO_RZ	DDD		CAEGGF								CAGSGRE			40
SSOMIO_RS	PRP		CAEGGF						APAN		CARSGRS			40
SSOMIS_R4	PCP		CAEGGF						CAPEN		CARSGRG			40
ScoMT8 Répart	CPP		CADUCS						NDEN		CAISGRA			33
PmiMT10 p1	MNP	CW	CAETCO						יאריםאר	N N	CATSCE	C		40
pmiMT10_C1	000	CW	CAECCP				C S N	R R	CAPCN	N N	CACSCRE	20		40
pmiMT10 D3	א שי קיק ק		CAEGGP		יציייי מח <mark>ר</mark>		C S P C	R R	CADAN	N N	CAKGGVG	20		40
PmiMT10_N3	PEA	CK	CAEGGP	C R				K	CAPEN	C K	CAKSGKG	CC		40
PmiMT10_R4	PCP	CK	CAEGGP						CADAN	A D	CAKGGKG			40
PmiMT10_R5	יד פי בי קיד קיד	CK	CAEGGP		CNE				ADEN	A D	CAKSGKO	00		40
PmiMT10 R7part	PGP	CW	CAEGGP							<u> </u>				16
RIGMM1 P1	MND		CKNSCV			с т п	CSK	N	יאידיכור	N N	CSTGGYC	C		43
BIOMTI DO	VGP	CN	CKETCN		CEK			N	CNPET	C K	CGSSDKA	CC	SSSD	44
B]eMT1 P3	VKP	CN	CKETCK	C H	CEK		CSK	N	אתפאר	CK	$C_{A} - SC$	CC	VGTT	41
BleMT1 R4	VEP	CN	CKETGK	Сн						CK	$C_{D} - S_{G}$		ASAT	41
BLeMT1 P5	VCP	CN	CKETGR	C H							C G = S N = -			41
BLeMT1 R6	VGP	CN	CKETGD	C H							CWTSNKC			40
BleMT1 R7	TSS	Сн	CISCOD	CR	CSD	SVD		T	CDPSK				Δ	40
BLeMT2 R1	MNP	CD	CKNSGV	Сн					CDI DI OI		CATGGKN		AAV-	43
BleMT2 R2	VGP	CN	CKETGX	Сн	CEK	CVD		N	CNPGT	CK	CASSNKA		TGCG	44
BLeMT2 R3	VGP	CN	CKETGN	Сн	CEK			N			CT-SG		TA	39
BleMT2 R4	MGP	CN	CKETGK	Сн	COK	CTD		N		CK	ст - SG			38
BleMT2 R5	AGP	C X	CKETGK	CL	CEK	стр	CSK	N		CK	$C_T - SG$		та	39
BleMT2 R6	MGP	CN	CKVTGK	Сн				N	CANS	CK	CWTSSKG			40
BleMT2 R7	NTP	Сн	CISCON	CR	CSD	SXD		т.	C D P F K		CVMSHOV		A	40
PmamiMT3 R1	MNP	CK	CAETGS	CR				R		CK	CSTSGKG			40
PmamiMT3 R2	OGP	CK	CAEGGP	Сн				K	APGN	CK	CAGSGKE			40
PmamiMT3_R3	RRP	CK	CAEGGP	Сн				E	APAN	CK	CAKSGKS			40
PmamiMT3 R4	PEA	CK	CADGGP	CR	CDK	CTD		ĸ	APEN	CK		CC		40
PmamiMT3_R5	PGP	СК	CAEGGP	Сн	CNE	Стр	csoc	ĸ	CAPAN	СК	CSESGKS	cc		40
PmamiMT3_R6	PGP	СК	CAEGGP	Сн	CNK	стр	CSRC	E	CAPEN	ск	CAKSGKG	c c		40
PmamiMT3 R7	PVP	СК	CAEGGP	Сн	CNK	стр	csoc	ĸ	CAPEN	ск	CAKSGKG	c c		40
PmamiMT3 R8	PGP	СК	CAEGGP	Сн	CDO	стр	csoc	к	CAPGN	ск	CATSGKA	c c		40
PmamiMT3 R9	GRP	CE	CADTGE	CR	CNK	стр	csoc	R	CNPKN	ск	CATAGKR	c c	RGTA	44
PauMT10 R1part		<u> </u>	CAETGS	CR	CDR	стр	csoc	0		ск	CATSGKG	c c		35
PauMT10 R2	OGP	ск	CAEGGP	Сн		стр	CSNC	ĸ	CAPGN	ск	CAGSGKE	c c		40
PauMT10_R3	PRP	СК	CAEGGP	Сн	CDE	стр	CSRC	к	CAPAX	ск	CAKSGKS	cc		40
PauMT10 R4	PEA	СК	CAEGGP	CR	CDK	CTD	CSRC	ĸ	CAPEN	ск	CAKSGKG	cr		40
PauMT10 R5	PGP	ск	CAEGGP	Сн	CDE	CTD	csoc	ĸ	CAPAN	ск	CAKSGKS	cc		40
PauMT10_R6	PEP	сĸ	CAEGGP	Сн	C N X	CTD		K	CAPEN	ск	CAKSGKG	c c		40
PauMT10_R7	PGP	сĸ	CAEGGP	Сн	cxo	CTD	csoc	K	CAPGN	ск	CATSGKA	c c		40
PauMT10_R8	GRP	CA	CADTGS	C R	CDK	CTD		R	CNPEN	сĸ	CATAGKO	c c	RGKA	44
BscMT1_R1	MNP	CD	<b>C</b> K N T G D	CQ	CAG	<mark>c</mark> g d d		N	DPAL	сĸ	~ C S T S A K T	c c		40
BscMT1_R2	APS	C N	<mark>C</mark> K E T G K	CQ	<mark>с</mark> ет (	CAD	csĸ	N C	C D P N L	сĸ	<mark>C</mark> TSAKKS	c c	SADA	44
BscMT1_R3	AGP	C N	<mark>C</mark> K E T G Q	СL	<mark>c</mark> sn <mark>o</mark>	<mark>c</mark> sd	c s n <mark>c</mark>	N C	CDPSL	c ĸ	<mark>C</mark> A S A E K A	c c		40
BscMT1_R4	AGP	CN	<mark>C</mark> KVTGK	СL	C V N (	C V D (	сттс	N C	CDPNL	сĸ	<mark>с</mark> тѕаккѕ	<mark>c c</mark>	FAEA	44
BscMT1_R5	VGP	CN	CKETGH	СL	<mark>с</mark> s n <mark>(</mark>	CSD	cssc	N	CNPSL	сĸ	<mark>C</mark> A S L E K A	c c		40
BscMT1_R6	SGP	C N	<mark>C</mark> K E T G H	СL	<mark>c</mark> s n <mark>(</mark>	<mark>c</mark> sd	css	N C	C D P S L	<mark>с</mark> к	<mark>C</mark> A S L E K A	c c		40
BscMT1_R7	SGP	C N	<mark>C</mark> KVTGV	СH	C A N <mark>(</mark>	C V D <mark>(</mark>	<mark>с т n с</mark>	N C	CDPAK	<mark>C</mark> G	<mark>C</mark> – S S D K G	<mark>c c</mark>	SASA	43
BscMT1_R8	ASP	C N	<mark>C</mark> K E T G N	C R	C D T <mark>(</mark>	<mark>c</mark> sd	<mark>с</mark> s n <mark>c</mark>	N C	CGLA-	<mark>с</mark> к	<mark>C</mark> S A A N K G	c c		39
BscMT1_R9	YAP	CI	C R T S G K	CQ	<mark>C</mark> M N <mark>(</mark>	C T D ·	<mark>-</mark> <mark>c</mark>	s	C D Q I T	<mark>C</mark> G	<mark>C</mark> P M V K V R			35
PpoMT7_R1	MNP	<mark>C</mark> K	<mark>C</mark> A E T G S	CQ	C D R <mark>(</mark>	C T D <mark>(</mark>	c s q <mark>c</mark>	R	C N P D N	C K	<mark>C</mark> A T S G K G	c c		40
PpoMT7_R2	QGP	C K	<mark>C</mark> A E G G P	Cн	C D Q <mark>(</mark>	C T D <mark>(</mark>	C S N C	K (	C A P G N	C K	<mark>C</mark> A G S G K E	c c		40
PpoMT7_R3	PRP	<mark>C</mark> K	<mark>C</mark> A E G G P	Cн	<mark>C</mark> D E <mark>(</mark>	C T D <mark>(</mark>	c s k <mark>c</mark>	E	C A P A N	C K	<mark>C</mark> A K S G K S	c c		40
PpoMT7_R4	PEA	<mark>C</mark> K	<mark>C</mark> A E G G P	C R	<mark>C</mark> D K <mark>(</mark>	C T D <mark>(</mark>	<mark>c</mark> s r <mark>c</mark>	K (	CAPEN	C K	<mark>C</mark> A K S G K G	c c		40
PpoMT7_R5	PGP	C K	<mark>C</mark> A E G G P	СH	<mark>C</mark> N E <mark>(</mark>	CTD	C N Q C	Q	<mark>C</mark> A P A N	C K	<mark>C</mark> A K S G K S	C C		40
PpoMT7_R6	PGP	C K	C A E G G P	CY	C N K (	CTD	c s q <mark>c</mark>	K	CAPEN	C K	<mark>C</mark> S K A G K G	c c		40
PpoMT7_R7	PGP	C K	C A E G G P	Cн	C N K (	CTD	c s q <mark>c</mark>	K	CAPEN	C K	C A K S G K G	c c		40
PpoMT7_R8	PGP	CK	C A E G G P	Сн	C D Q (	CTD	c s q <mark>c</mark>	K	C A P G N	CK	C A T S G K A	C C		40
PpoMT7_R9	- G P	CA	<mark>C</mark> A D T G S	C R	C D K (	CTD	c s q <mark>c</mark>	R	<mark>C</mark> NPKN	C R	<mark>C</mark> A T A G K Q	c c	RGKA	43
		Сx	Cxxxxx	Cx	Cxx(	Cxx(	CxxC	$\mathbf{x}$	Cxxxx(	$\mathbf{C}\mathbf{x}$	Cxxxxxx	CC		
						1	20-		2					
							2 U _	Z 🛱						

**Figure S3. Amino acid alignment of multimodular Stelydae repeats of Ascidian MTs.** Conserved cysteines are highlighted in yellow. Configurations of cysteine motifs are showed below each alignment. Names of MT sequences from different species are as in Supplemental\_Table\_S1.pdf.

**Figure S4** 



**Figure S4.** ESI-MS spectra recorded at neutral pH showing the corresponding charge state (+3 or +4) of CroMT1, HroMT1, HroMT2 and BscMT1 (R4) synthesized in Zn-enriched media (A, C, E and G, respectively), or in Cu-enriched media (B, D, F and H, respectively). Glycosylated metallated-species are marked with asterisk (\*).



**Figure S5**. ESI-MS spectra recorded at neutral pH showing the corresponding charge state (+3 or +4) of SthMT1, SthMT2, SthMT3 and SthMT4 synthesized in Zn-enriched media (A, B, C and D), or SthMT1, SthMT3 produced in Cu-enriched media (E and F). Glycosylated metallated-species are marked with asterisk (\*).

Figure S6



**Figure S6. Amino acid alignment of Appendicularia MTs**. Conserved cysteines are highlighted in yellow. Configurations of cysteine motifs are showed below the alignment. Names of MT sequences from different species are as in Supplemental\_Table\_S1.pdf. (A) Alignment of OdiMT1 with the six repeats units (RU1 to 6) of OdiMT2. Notice that a RU is made of two C7 subunits (C7a and C7b) plus a carboxyl-terminal tail with 5 cysteines (C5). Comparison of OdiMTs with MTs from other *Oikopleura* species revealed that appendicularian MTs were multi-modular proteins made of a conserved 12-Cys domain that corresponded to the previously described C7b+C5 subunits (see text for details). OdiMT1 would be therefore a bi-modular MT, whose first 12-Cys domain (formerly known as C7a) would be a trimmed 12-Cys domain (t-12C) lacking the five last cysteines (C5). OdiMT2 would be a multi-modular MT made of twelve 12-Cys domains, whose odd domains would have been also trimmed as t-12C. (B) Alignment of the C12 domains of several multi-modular appendicularian MTs.



**Figure S7. Cephalochordate gene structure.** Cephalochordate *MT1* genes have four exons: exons 1 and 2 codify for the 11 Cys domain (blue box), exon 3 codifies for the 9  $Cys_{Like}$  domain (red box), and exon 4 codifies for the carboxyl-terminal end devoid of Cys residues. Cephalochordate *MT2* genes have four exons, a tiny exon 1, followed by three similar exons (exon 2, 3 and 4), each one encoding for a 9-Cys domain (red box). Exon 2 is included in three-domain MT2L isoforms but skipped in the two-domain MT2S isoforms.

#### Figure S8



**Figure S8. Amino acid alignment of Cephalochordate MTs**. Conserved cysteines are highlighted in yellow. Configurations of cysteine motifs are showed below each alignment. Names of MT sequences from different species are as in Supplemental\_Table\_S1.pdf, and alphabetically ordered. (A) Cephalochordate MT1 alignment. (B) Cephalochordate MT2L alignment. (C) Alignment of 9-Cys domains of MT2s and 9-Cys<sub>Like</sub> domains of MT1s.

<b>Figure</b>	e <b>S9</b>				-					4.0				<u></u>		
•					4	:0 				40 				60 I		
AcaMT3	MDPQA	s	ATGG	S <mark>Y</mark> N <mark>(</mark>	AGS	<mark>c k c</mark> k :	р <mark>скс</mark> т	з <mark>с</mark> ккя	s <mark>cc</mark> sc	<mark>C</mark> PAS	S D N C	AKGC	V <mark>C</mark> KDPS	S G K	<mark>c s c c</mark> x	63
AcaMT4	M D A Q D <mark>(</mark>	C P C	ISGG.	а <mark>с</mark> т <mark>о</mark>	GNN	<mark>с q с</mark> кі	N <mark>C</mark> KCK	т <mark>с</mark> ккя	s <mark>ccs</mark> c	<mark>C</mark> P V G	<mark>c</mark> sk <mark>c</mark>	AQG <mark>C</mark>	I <mark>С</mark> КСРР	ssk	с <mark>sсс</mark> к	63
BtaMT1A	MDP-N <mark>(</mark>	s s	PTGG	s <mark>c</mark> s <mark>c</mark>	AGS	<mark>стс</mark> к:	a <mark>c</mark> r <mark>c</mark> p	S <mark>C</mark> KKS	s <mark>cc</mark> sc	<mark>C</mark> P V G	<mark>c</mark> ak <mark>c</mark>	A Q G <mark>C</mark> V	/ <mark>C</mark> KGA	SDK	C S C C A	61
BtaMT1E	MDP-N <mark>(</mark>	s s	STGG	s <mark>c</mark> s <mark>c</mark>	C P G S <mark>(</mark>	<mark>стс</mark> к:	a <mark>c</mark> r <mark>c</mark> p	SCKKS	s <mark>cc</mark> sc	<mark>C</mark> P V G	<mark>C</mark> A K <mark>C</mark>	AQG <mark>C</mark> :	I <mark>C</mark> KGA – – –	sdk	C S C C A	61
BtaMT1E2	MDP-N <mark>C</mark>	c s c	PTSG	s <mark>c</mark> s	CAGS	<mark>стс</mark> к:	a <mark>c</mark> r <mark>c</mark> p	S C K K S	s <mark>cc</mark> sc	<mark>C</mark> P V G	<mark>C</mark> A K <mark>C</mark>	AQG <mark>C</mark> :	I <mark>C</mark> KGA – – –	SDK	C R C H A	61
BtaMT2A	MDP-N <mark>C</mark>	s s	TAGE	S <mark>С</mark> Т <mark>О</mark>	CAGS	<mark>C K C</mark> K :	d <mark>c k c a</mark>	S C K K S	s <mark>cc</mark> sc	<mark>C</mark> P V G	<mark>C</mark> A K <mark>C</mark>	AQG <mark>C</mark> V	7 <mark>C</mark> KGA – – –	S D K	C S C C A	61
BtaMT3	MDPET	C P C	PTGG	S <mark>C</mark> T <mark>(</mark>	SDP	C K <mark>C</mark> E (	G <mark>C T C </mark> A	S <mark>S</mark> K K S	s <mark>ccs</mark> c	<mark>C</mark> P A E	C E K C	AKD <mark>C</mark> V	V <mark>C</mark> K G G E G A	EAEEKK	<mark>c s c c</mark> o	68
BtaMT4	MDSGE	C T C	MSGG	r <mark>ca</mark> (	G D N <mark>(</mark>	C K <mark>C</mark> T '	т <mark>сsс</mark> к	TCRKS	G <mark>CCP</mark> C	<mark>C</mark> P P G	C A K C	ARG <mark>C</mark>	I <mark>C</mark> KGA	SDK	C S C C P	62
CliMT1	MDPQD	C T C	CAAGD	s <mark>c</mark> s c	AGS	C K C K I	N C R C Q	SCRKS	s <mark>ccs</mark> c	C P A S	<mark>с s n c</mark>	AKGC	/ <mark>C</mark> KEPS	sk	сѕссн	63
EbuMT1	MSDTP	TC	NLGD	r <mark>c</mark> n (	GDN	CKCK:	D <mark>CKC</mark> T	SCKKS	SCCAC	C P A G	C S K C	A Q G C	V <mark>C</mark> KGA	SDK	<mark>c s c c</mark> Q	62
GgaMT3	MDSQD	C P C	ATGG	г <mark>ст</mark>	GDN	C K C K I	N C K C T	SCKKO	a <mark>ccsc</mark>	C P A G	CAKC	A Q G C	V <mark>C</mark> KGPP	SAK	<mark>с s c c</mark> к	63
GgaMT4	MDPQD	TC	AAGD	scso	CAGS	CKCKI	NCRCR	SCRKS	a c c s c	CPAG	CNNC	AKGC	V <mark>C</mark> KEPA	S S K	сѕссн	63
HSaMTIA	MDP - NC		ATGG	SCTO	TGS	CKCK	ECKCT	SCKKS	SCCSC	C P M S	CAKC	AQGC	[ <b>C</b> K G A	SEK	CSCCA	61
HsaMT3	MDPET		PSGG	SCTO			GCKCT	SCKKS		CPAE	CEKC	AKDCV	CKGGEAA	EAEAEK		68
HsaM14	MDPREC				GDN		TCNCK	TYWKS		C P P G	CAKC	ARGC	C K G G	SDK		62
HSAMTIB								SCKKC			CAKC	AQGC	VCKGS	SEK		61 61
HSAMTIE			ATGG				ECKCT	SCKKS			CAKC	AQGC	CKGA	SEK		61
HSaMIIF	MDP = NC				AGS			CVVC			CAVC	AUGU	CKGA	SER		61
nsamiig UcoMT1U	MDP = NC		TEACC		ASS			C V V C			CARC	AQGC.	CKGA	SER		61
HeaMT1M								SCKKS			CARC	AUGC		SER		61
HsaMT1X	MDP-N		SPVG		AGS			SCKKS		CPVG	CAKC	AOGC			CSCCA	61
HsaMT2A	MDP-NO		CAAGD	SCT	AGS	CKCK	ЕСКСТ	SCKKS	s c c s c	CPVG	CAKC	AOGC	C K G A	SDK	CSCCA	61
MmuMT1	MDP-N	csc	STGG	SCT	TSS	CACKI	NCKCT	SCKKS	sccsc	CPVG	CSKC	AOGC	VCKGA	ADK	CTCCA	61
MmuMT2	MDP-N	s	ASDG	scs		CKCK	о <mark>скс</mark> т	SCKKS	sccsc	CPVG	CAKC	SOGC	I <mark>С</mark> КЕА	S D K	CSCCA	61
MmuMT3	MDPET	C P C	PTGG	S С Т (	SDK	CKCK	с скст	NCKKS	sccsc	CPAG	CEKC	AKDC		KAEAEK	cscco	68
MmuMT4	MDPGE	стс	MSGG	ICIC	GDN	скст	т <mark>сѕс</mark> к	TCRKS	сс <mark>рс</mark>	<mark>с</mark> ррд	CAKC	ARGC	г <mark>с</mark> кдд	sdk	c s c c P	62
OlaMT1	MDP <mark>(</mark>	D D C	SKTG	к <mark>с</mark> м	GGS	ст <mark>с</mark> ті	и <mark>с</mark> ѕст	s <mark>c</mark> kks	CCAC	<mark>C</mark> P S G	стк <mark>с</mark>	ASGC	и <mark>с</mark> кдк – – –	тср <mark>'</mark>	т <mark>тсс</mark> д	60
PsiMT1	MDPQD	C A C	ATGA	s <mark>с</mark> т <mark>с</mark>		<mark>c k c</mark> k i	и <mark>скс</mark> т	SCKKS	s <mark>cc</mark> sc	<mark>C</mark> P A G	<mark>c</mark> ak <mark>c</mark>	AKSC	V <mark>C</mark> KEPL	SDK	с <mark>sсс</mark> т	63
RnoMT1A	MDP-N <mark>(</mark>	so	STGG	s <mark>с</mark> т(	sss	с <mark>с</mark> кі	и <mark>скс</mark> т	s <mark>c</mark> kks	s <mark>cc</mark> sc	<mark>C</mark> P V G	<mark>c</mark> sk <mark>c</mark>	AQGC	/ <mark>C</mark> KGA – – –	SDK	C T C C A	61
RnoMT2A	MDP-N <mark>(</mark>	c s c	ATDG	s <mark>c</mark> s <mark>c</mark>	AGS	<mark>скс</mark> к	д <mark>скс</mark> т	s <mark>c</mark> kks	s <mark>cc</mark> sc	<mark>C</mark> P V G	<mark>c</mark> ak <mark>c</mark>	s Q G <mark>C</mark> :	I <mark>C</mark> KEA	sdk	C S C C A	61
RnoMT3	M D P E T <mark>(</mark>	C P C	PTGG	S <mark>С</mark> Т <mark>(</mark>	S D K <mark>(</mark>	C K C K (	с <mark>скс</mark> т	N <mark>C</mark> KKS	s <mark>cc</mark> sc	<mark>C</mark> P A G	<mark>c</mark> ek <mark>c</mark>	AKDC	7 <mark>C</mark> K G E E G A	K – – AEK	c s c c q	66
RnoMT4	M D P G E <mark>(</mark>	ст <mark>с</mark>	CMSGG	I <mark>CI</mark>	G D N <mark>(</mark>	<mark>скс</mark> т'	т <mark>с</mark> ѕск	TCRKS	б <mark>ССР</mark> С	<mark>C</mark> P P G	<mark>C</mark> A K <mark>C</mark>	ARG <mark>C</mark> I	I <mark>С</mark> КGG – – –	S D K	с <mark>sсс</mark> р	62
TguMT1	MDSQD	C P C	ATGG	г <mark>С</mark> Т <mark>(</mark>	G D N <mark>(</mark>	C K C K I	N <mark>C</mark> K <mark>C</mark> T	S C K K G	e <mark>ccs</mark> c	<mark>C</mark> P A G	<mark>C a k C</mark>	AQG <mark>C</mark> V	/ <mark>C</mark> KGPP	SAK	<mark>с s с с</mark> к	63
TguMT2	MDPQD	C T C	CAAGD	s <mark>c</mark> s <mark>c</mark>	C A G S <mark>(</mark>	C K C K I	N <mark>C</mark> R <mark>C</mark> R	SCRKS	s <mark>cc</mark> sc	<mark>C</mark> P A S	<mark>с</mark> s n <mark>c</mark>	AKG <mark>C</mark> V	/ <mark>С</mark> КЕРТ – –	sk	с <mark>sсс</mark> н	63
TniMTa	MDP C	DC	AKSG	S C T (	G G S <mark>(</mark>	C A C T I	N <mark>C</mark> A <mark>C</mark> T	TCKKS	G <mark>CCP</mark> C	<mark>C</mark> P S G	C S K C	ASG <mark>C</mark> V	/ <mark>C</mark> KGK	TCD	T S C C Q	60
TniMTb	MDP <mark>(</mark>	2 D C	C A K T G	N C K (	GGS	C T C K I	D <mark>C</mark> SCT	D C K K S	s <mark>ccs</mark> c	<mark>C</mark> P S G	<mark>с</mark> s к <mark>с</mark>	ASGC	/ <mark>C</mark> KGK – – –	TCD	TSCCQ	60
TruMT1F	MDP <mark>C</mark>	2 D C	SKTG	S C N C	GGS	C A C K I	N C S C T	TCKKS	s <mark>ccs</mark> c	<mark>C</mark> P S G	<mark>c</mark> sk <mark>c</mark>	ASGC	/ <mark>C</mark> KGK – – –	TCD	TSCCQ	60
XtrMT4	M D P Q D <mark>(</mark>	C N C	ETGA	s <mark>c</mark> s	ANK	cvcs:	N <mark>C</mark> KCT	SCKKS	s <mark>ccs</mark> c	C P A E	C N K C	SKG <mark>C</mark> I	H <mark>C</mark> EKE – – –	S K K	<mark>с s с с</mark> n	62
	C	'xC	XXXXX	(CxC	XXX	CxCx	xCxCx	хC	CCxC	Cxxx	CxxC	xxxC	xCxxxxxx	xxxxxx	CxCC	
				90	Cys							11	Cys			
				) n-									-			
				ັບເ	mal	11						α μ	Jinain			

**Figure S9. Amino acid alignment of vertebrate MTs.** Conserved cysteines are highlighted in yellow. Configurations of cysteine motifs are showed below each alignment. Names of MT sequences from different species are as in Supplemental\_Table\_S1.pdf, and alphabetically ordered.

Subphylum	Class	Order	Family	Species	MT name	Accession Number	Sequence	K/N ratio
/ERTEBRATES								
	Mammalia							
		Artiodactyla	Bovidae	Bos taurus	BtaMT1A	XP_024833736.1	MDPNCSCPTGGSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCVCKGASDKCSCCA	6:1
					BtaMT1E	NP_001108329.1	MDPNCSCSTGGSCSCPGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCICKGASDKCSCCA	6:1
					BtaMT1E2	NP_001071602.1	MDPNCSCPTSGSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCICKGASDKCRCHA	6:1
					BtaMT2A	NP_001068608.1	MDPNCSCTAGESCTCAGSCKCKDCKCASCKKSCCSCCPVGCAKCAQGCVCKGASDKCSCCA	8:1
					BtaMT3	NP_001106775.1	MDPETCPCPTGGSCTCSDPCKCEGCTCASSKKSCCSCCPAECEKCAKDCVCKGGEGAEAEEKKCSCCQ	8:0
					BtaMT4	NP_001108331.1	MDSGECTCMSGGTCACGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKGASDKCSCCP	6:1
		Primates	Hominidae	Homo Sapiens	HsaMT1A	NP_005937.2	MDPNCSCATGGSCTCTGSCKCKECKCTSCKKSCCSCCPMSCAKCAQGCICKGASEKCSCCA	8:1
					HsaMT1B	NP_005938.1	MDPNCSCTTGGSCACAGSCKCKECKCTSCKKCCCSCCPVGCAKCAQGCVCKGSSEKCRCCA	8:1
					HsaMT1E	NP_783316.2	MDPNCSCATGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKGASEKCSCCA	8:1
					HsaMT1F	NP_005940.1	MDPNCSCAAGVSCTCAGSCKCKECKCTSCKKSCCSCCPVGCSKCAQGCVCKGASEKCSCCD	8:1
					HsaMT1G	NP_005941.1	MDPNCSCAAGVSCTCASSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASEKCSCCA	8:1
					HsaMT1H	NP_005942.1	MDPNCSCEAGGSCACAGSCKCKKCKCTSCKKSCCSCCPLGCAKCAQGCICKGASEKCSCCA	9:1
					HsaMT1M	NP_789846.2	MDPNCSCTTGVSCACTGSCTCKECKCTSCKKSCCSCCPVGCAKCAHGCVCKGTLENCSCCA	6:2
					HsaMT1X	NP_005943.1	MDPNCSCSPVGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGTSDKCSCCA	8:1
					HsaMT2A	NP_005944.1	MDPNCSCAAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKGASDKCSCCA	8:1
					HsaMT3	NP_005945.1	MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ	8:0
					HsaMT4	NP_116324.2	MDPRECVCMSGGICMCGDNCKCTTCNCKTYWKSCCPCCPPGCAKCARGCICKGGSDKCSCCP	6:2
		Rodentia	Muridae	Mus Musculus	MmuMT1	NP_038630.1	MDPNCSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKGAADKCTCCA	7:2
					MmuMT2	NP_032656.1	MDPNCSCASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKEASDKCSCCA	8:1
					MmuMT3	NP_038631.1	MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ	11:1
					MmuMT4	NP_032657.1	MDPGECTCMSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKGGSDKCSCCP	6:1
				Rattus norvegicus	RnoMT1A	NP_620181.1	MDPNCSCSTGGSCTCSSSCGCKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKGASDKCTCCA	7:2
					RnoMT2A	NP_001131036.1	MDPNCSCATDGSCSCAGSCKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKEASDKCSCCA	8:1
					RnoMT3	NP_446420.1	MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEKCSCCQ	11:1
					RnoMT4	NP_001119556.1	MDPGECTCMSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKGGSDKCSCCP	6:1
	Aves							
		Galliformes	Phasianidae	Gallus gallus	GgaMT3	NP_001091007.1	MDSQDCPCATGGTCTCGDNCKCKNCKCTSCKKGCCSCCPAGCAKCAQGCVCKGPPSAKCSCCK	9:2
					GgaMT4	NP_990606.1	MDPQDCTCAAGDSCSCAGSCKCKNCRCRSCRKSCCSCCPAGCNNCAKGCVCKEPASSKCSCCH	6:3
		Columbiformes	Columbinae	Columba livia	CliMT1	P15787	MDPQDCTCAAGDSCSCAGSCKCKNCRCQSCRKSCCSCCPASCSNCAKGCVCKEPSSSKCSCCH	6:2
		Passeriformes	Estrildidae	Taeniopygia guttata	TguMT1	NP_001232450.1	MDSQDCPCATGGTCTCGDNCKCKNCKCTSCKKGCCSCCPAGCAKCAQGCVCKGPPSAKCSCCK	9:2
					TguMT2	NP_001232079.1	MDPQDCTCAAGDSCSCAGSCKCKNCRCRSCRKSCCSCCPASCSNCAKGCVCKEPTSSKCSCCH	6:2
	Reptilia							
		Squamata	Dactyloidae	Anolis carolinensis	AcaMT3	H9GCQ4	MDPQACSCATGGSYNCAGSCKCKDCKCTSCKKSCCSCCPASSDNCAKGCVCKDPSSGKCSCCX	8:2
					AcaMT4	H9GQC7	MDAQDCPCISGGACTCGNNCQCKNCKCKTCKKSCCSCCPVGCSKCAQGCICKGPPSSKCSCCK	9:3
			Lacertidae	Podarcis siculus	PsiMT1	Q708T3	MDPQDCACATGASCTCAGSCKCKNCKCTSCKKSCCSCCPAGCAKCAKSCVCKEPLSDKCSCCT	9:1
	Amphibia							
		Anura	Pipidae	Xenopus tropicalis	XtrMT4	NP_001165150.1	MDPQDCNCETGASCSCANKCVCSNCKCTSCKKSCCSCCPAECNKCSKGCHCEKESKKCSCCN	9:5
	Actinopterygii							
		Beloniformes	Adrianichthyidae	Oryzias latipes	OlaMT1	NP_001098255.1	MDPCDCSKTGKCNCGGSCTCTNCSCTSCKKSCCACCPSGCTKCASGCVCKGKTCDTTCCQ	7:2
		Tetraodontiformes	Tetraodontidae	Takifugu rubripes	TruMT1F	XP_003967042.1	MDPCDCSKTGSCNCGGSCACKNCSCTTCKKSCCSCCPSGCSKCASGCVCKGKTCDTSCCQ	7:2
				Tetraodon nigroviridis	TniMTa	Q4SKC8	MDPCDCAKSGSCTCGGSCACTNCACTTCKKSCCPCCPSGCSKCASGCVCKGKTCDTSCCQ	6:1
				-	TniMTb	Q4SKC7	MDPCDCAKTGNCKCGGSCTCKDCSCTDCKKSCCSCCPSGCSKCASGCVCKGKTCDTSCCQ	8:1
	Myxini							
		Myxiniformes	Myxinidae	Eptatretus burgeri	EbuMT1	SRA	MSDTPCTCNLGDTCNCGDNCKCKDCKCTSCKKSCCACCPAGCSKCAQGCVCKGASDKCSCCQ	8:3
		-	-					

CEPHALOCHORDATES

Leptocardii

1							
	Amphioxiformes	Asymmetronidae	Asymmetron lucayanum	AluMT1 AluMT2 (S/L)	SRA SRA	MPDPCNCAQTGTCSCAGQTCGCGDHCECGDGCKCVGCKMHGNVVVTCCGDCTGIGKNCACGCSCCQPDVPAVTISTPPAAHL MPDPCCAACKGCGLASCNCGCDCCKCCDACPGVCGPDCACGCSCCKKCASCDSCNSCKPGCTTCSCDCCK MPDPCNCAQSGACSCAGGTCQCGDDCRCGDGCKCVGCKLHGNVSVTCCAMCTGKVKNCACGCSCCQPAAPAVVNMTTPPAA	3:3 6:2 H
		Branchiostomidae	Branchiostoma belcheri	BbeMT1 BbeMT2 (S/L)	XP_019631158 JZ816971	L MPDPCCADCKGCGSAGCKCGCDCCKCCASCPGCGPGCNSCGCDCCKCCASCDSCKSGCTTCSCDCCK	4:4 6:1
			Branchiostoma japonicum	BjaMT1 BjaMT2 (S/L)	SRA SRA	MPDPCNCAQSGACSCSGGTCQCGDDCQCGDGCKCVGCKLHGNVNVTCCGDCTGIGKNCACGCSCCLPAAPAVTTMTTPPAAH MPDPCCADCKGCGSAGCKCGCDCCKCCASCTGCNPGCTSCSCDCCKCCASCDGCKSGCTTCSCDCCK	3:4 6:1
			Branchiostoma lanceolatum	BlaMT1 BlaMT2 (S/L)	JT862963 JT872034	MPDPCNCAQSGACSCNGLCQCGDDCQCGDGCKCVGCKLHGNVDVTLTCCGTCTGIGKNCACGCSCCQPDVPAVTVLTTPPAAH MPDPCCSACTGCSTSCKSCNCDCCKCCASCTGCSPNCNSCGCDCCKCCASCDGCKSGCTSCSCDCCK	L 3:4 5:3
			Branchiostoma floridae	BfIMT1 BfIMT2(S/L)	SRA BW841405	MPDPCNCAQSGTCSCGGPCQCGDDCQCGDGCKCVGCKLHSNVTDIVTCCVDCKGIGKNCACGCSCCQPDTPAVAILTTPPAAHL MPDPCCSACEGCSSTCNKCSCDCCKCCASCKACGPTADCSCGCACCKCCASCDGCKSSCTSCSCDCCK	4:3 6:1
TUNICATES							
Ascidiacea							
	Aplousobranchia	Clavelinidae	Clavelina lepadiformis	CleMT1	SRA	MDPCNCAETGVCKCENCTDCSKCNCNTKTCKCTKSCCPK	6:4
		Polycitoridae	Cystodytes dellechiajei	CdeMT1	SRA	MDPCKCAETGGDCCCSNCSDCSNCKCNPDKCKCSGGCCKK	6:3
		Holozoidae	Distaplia occidentalis	DocMT1	SRA	MDPCNCAETGVCKCTGCNDCSNCKCNPKTCKCTKGCSGCKE	6:4
				DocMT2	SRA	MDPCNCTETGVCHCDKCTDCTNCKCLPTNCMCTQGKCTQSKCSCANVCKK	6:4
	Phlebobranchia	Ascidiidae	Ascidia sp T160	AspMT1	SRA	MDPCKCAETGVCTCDQCKDCSNCKCNPSLCKCKKSGCGSCSKK	8:2
				AspMT2	SRA	MDPCNCSDTGVCKCXQCKDCSNCKCNPSLCKCSGKGCGSCSKK	7:3
				AspMT3	SRA	MDPCNCVGTGKCHCNQCDDCSKCKCNPDVCKCAKSKKSCCPSK	8:3
			Phallusia fumigata	PfuMT1	SRA	MDPCNCAETGTCKCDQCKDCSKCKCNPATCKCGGGCCK	6:2
				PfuMT2	SRA	MDPCNCSTTGVCKCDQCKDCSNCKCGSSSCKCASGCCK	5:2
			Phallusia mammilata	PmammMT1	SRA	MDPCKCAETGNCTCLECKDCSKCKCNPENCKCNRPCCKK	7:4
		Cionidae	Ciona robusta <sup>1</sup>	CroMT1	ACN32211	MDPCNCAETGVCNCVDCSNCSSCNCDPKICNCAKACCPK	3:5
			Ciona intestinalis <sup>2</sup>	CintMT1	SRA	MDPCNCAATGVCKCEGCTDCKNCNCDPKVCKCTKSCCPK	6.3
			Ciona intestinaiis	CintMT2	SRA		6.3
			Ciona savignvi	CsaMT1	SRA		6.2
		Corellidae	Corella inflata	CinfMT1	SRA	MDPCKCSETGVCHCDKCTDCTNCKCDSSKCKCAGAKKACCGA	7.1
		coremade	Corella willmeriana	CwiMT1	SRA	MDPCNCAATGVCKCEGCTDCSKCKCNPETCCASKGCCK	5.2
				CwiMT2	SRA	MDPCNCAETGVCKCTGCNDCSNCKCNPKTCKCTKGCSGCKE	6:4
				CwiMT3	SRA	MDPCNCAATGVCKCEGCTDCSKCKCNPETCCASKGCCK	5:2
	Stolidobranchia	Molgulidae	Mogula occidentalis	MocciMT1	\$539075	MDPCNCADTGNCHCVDCTDCSKCNCNPAICKCTKPCCPK	4:4
		0	Mogula occulta	MoccuMT1	SRA	MDPACNCAETGKCTCDGCTSCTNCKCNPETCKCAAGCCKK	5:3
				MoccuMT2	SRA	MDPCNCATTGNCHCDTCSDCSKCNCNPTNCICNKSCCKK	4:6
			Mogula oculata	MocuMT1	S112679	MDPEACNCAETGQCTCENCTNCKCNPETCKCGAGCCKK	4:5
				MocuMT2	S94991	MDPCNCAATGNCHCDKCTDCSKCNCNPTNCICNRSCCKK	4:6
				MocuMT3	S103250	MDPCNCAETGNCHCDKCTDCSKCSCNPTNCICNRSCCEK	3:5
		Pyuridae	Halocynthia aurantium	HauMT1	SRA	MDPCKCSETGVCRCNDCTNCSKCKCDPVLCNCRKDSKQCCGK	6:3
				HauMT2	SRA	MDPCKCSETGVCRCDNCKDCSNCKCDPTLCKCKKDSKECCGK	8:2
			Halocynthia roretzi	HroMT1	SRA	MDPCKCSETGVCRCNDCTNCSKCKCDPALCNCRKGSKQCCGK	6:3
				HroMT2	SRA	MDPCKCSETGVCRCDNCKDCSNCKCDPTLCKCKKESKECCGK	8:2
			Herdmania curvata	HcuMT1	AY314949	MDPCNCAETGVCRCVGCTDCSKCNCNPSTCKCSSGGKKCCS	4:3
			Microcosmus sabatieri	MsaMT1part	SRA	MNPCECAETGSCRCDRCTDCSQCRCNPDNCKCSTSGKGC	2:3
				MsaMT2	SKA		6:5
			Duran dura	MsaMT3	SKA		8:1
			Pyura dura	POUMI1	SKA		8:2
				PUUIVI I Z	SUN		8:2 7.0
				POUIVII3	SKA		/:b
1				Paulvi 14	SKA	MIDPUNUKE I GVUKUDUUSDU I KUNUNDI UKUSKASKSUUPSG I K	6:3

Stvelidae	Asterocarpa humilis	AhuMT1	SRA	MDPCNCKDTGVCNCDKCTDCSKCNCNPDVCKCAKAKKSCCPK	8:4
		AhuMT2	SRA	MDPCNCAVSGNCHCEOCDDCXGCKCNPDVCKCAKSKKSCCASK	6:3
		AhuMT3	SRA	MDPCNCAETGVCNCNNCTDCSNCKCDPAVCKCSKASKTCCPTK	5:5
		AhuMT4	SBA	MDPCNCAFTGNCHCNOCDDCSKCKCNPSVCKCAKSSKSCCPSK	6.4
		AhuMT5	SBA	MDPCNCVGTGKCHCNOCDDCSKCKCNPDVCKCAKSKKSCCPSK	8.3
		AhuMT6	SBA	MDPCNCKETGVCHCDOCTDCSKCNCNPNTCKCSSAKSCCSK	6.4
		AbuMT7	SRA		6.7
		AbuMT7part	SRA		0.2 1·1
		Andiviri/part	3174		
				SSSDVKPCNCKETGKCHCEKCTDCSKCNCNPDNCKCASGCC/GTTVEPCNCKETGKCHCDKCTDCSKCNCNPDNCKCDSGCCASG	т
					, c
	Botrylloides leachii	BIoMT1	SRA		20.26
	bott yiloides leachin	DIEWITI	3174		20.20
					c
					28:23
		PIONT2	SDA.		
		DIEIVITZ	ATSW01006707 1		
			A13W01000707.1,		
				MNPCDCkNTGDCQCAGCGDCSGCNCDPALCKCSTSAKTCCAPSCNCKETGKCQCETCADCSKCNCDPNLCKCTSAKKSCCSADAA	<b>۱</b>
			JG326099.1	GPCNCKETGQCLCSNCSDCSNCNCDPSLCKCASAEKACCAGPCNCKVTGKCLCVNCVDCTTCNCDPNLCKCTSAKKSCCFAEAVGP	
	Botryllus schlosseri	BscMT1	CCA015503.01°;	CNCKETGHCLCSNCSDCSSCNCNPSLCKCASLEKACCSGPCNCKETGHCLCSNCSDCSSCNCDPSLCKCASLEKACCSGPCNCKVTGV	/ 31:31
			JG321680.1	CHCANCVDCTNCNCDPAKCGCSSDKGCCSASAASPCNCKETGNCRCDTCSDCSNCNCGLACKCSAANKGCCYAPCICRTSGKCQC	
			CCAU13237.D1*;	MNCTDCSCDQITCGCPMVKVR	
			JG326100.1 CCA015503.g1		
	Dendrodoa grossularia	DørMT1	SRA	MDPCNCKETGVCHCDRCTDCSKCNCNPDTCKCAKSSKSCCPTSK	6:3
	Denarouou grossularia	DgrMT2	SRA		6.2
		DgrMT2	SRA		7.2
		DgrMT4	SRA		5.3
		DgrMT5	SRA		6.3
		DgrMT6	SRA		5.3
		DEIWIN	JIA		5.5
		DgrMT7	SRA	MNPCTCADDGPCHCDQCTDCSSCRCSSANCKCATSGKSCCGGQTGEPCTCADSGPCRCAQCTECSRCRCSPANCQCSKSGRTCCV	3:3
	Distomus variolosus	DvaMT1	SRA	MDPCNCKDTGACHCVKCTDCTNCKCNPNTCKCAKANCCPK	6:5
		DvaMT2	SRA	MDPCNCKETGVCHCDKCTDCSKCSCDPNTCKCASAKKSCCQK	7:2
		DvaMT3	SRA	MDPCNCKETGVCHCDRCTDCSKCNCNPDTCKCAKSSKSCCPTSK	6:3
		DvaMT4	SRA	MDPCNCKETGVCKCDQCSDCSKCNCSPDTCKCTXANKSCCPSGTK	6:3
		DvaMT5	SRA	MDPCNCAETGICNCTQHADCGKCSCNPEVCKCSQAKKGCCGAK	5:3
		DvaMT6	SRA	MDPCNCKESGVCHCDKCTDCSKCACDIDNCKCANVKKTCCPTK	7:3
	Eusynstyela tincta	EtiMT1	SRA	MDPCNCTETGVCKCDQCDCSKCNCDPDTCKCTKAKKSCCPSGTK	7:2
		EtiMT2	SRA	MDPCNCAETGTCHCDQCTDCTKCKCNPETCKCSQAKKSCCTK	6:2
		EtiMT3	SRA	MDPCNCKDTGTCHCDQCTDCSKCNCNTAACKCSKPCCPK	5:3
		EtiMT4	SRA	MDPCNCKETGVCHCDKCTDCSKCNCNPETCKCQKSCCPK	6:3
		EtiMT5	SRA	MDPCNCAETGNCHCNQCDDCSKCKCNPSVCKCAKSKKSCCPSK	7:4
		EtiMT6	SRA	MDPCNCAETGVCNCNNCTDCSNCKCDPAVCKCSKASKTCCPTKETGIOCRTNDEVEIN	5:7
		EtiMT7	SRA	MDPCNCAVSGNCHCEQCDDCXGCKCNPDVCKCAKSKKSCCASK	6:3
				MNPCKCAETGSCRCDRCTDCSOCQCNPDNCKCATSGKGCCQGPCKCAEGGPCHCDOCTDCSNCKCAPGNCKCAGSGKECCPRPC	С
		EtiMT8part	SRA	KCAEGGPCHCDECTDCSRCKCAPANCKCAKSGKSCCPEACKCA	13:6
	Polyandrocarpa anguinea	PanMT1	SRA	MDPCNCKETGVCVCEKCTDCSKCNCNPNTCRCSENQKSCCH	4:5
		PanMT2	SRA	MDPCNCKETGLCHCDKCTDCSKCNCNPDICRCAKDQKSCCH	5:3
		PanMT3	SRA	MDPCNCKETGVCVCDKCTDCSRCNCNPDTCKCSENQKSCCPk	5:4
		PanMT4	SRA	MDPCNCKETGVCHCDKCTDCSKCNCNPDTCKCQKTCCPK	6:3
	Delver der er i dit i	D 11 4771	<b>CD A</b>		F. 0
	Polyandrocarpa misakiensis	PMINI1	SKA		5:2
		PMINI 2	SKA	Μυγενεκνισαεμευρεγαιρικά ματαγραφικά ματαγρ	5:2

	PmiMT3	SRA	MDPCNCAVSGNCHCEQCDDCXGCKCNPDVCKCAKSKKSCCASK	6:3
	PmiMT4	SRA	MDPCNCKDTGVCNCDKCTDCSKCNCNPDVCKCAKAKKSCCPK	8:4
	PmiMT5	SRA	MDPCNCAETGTCNCQNCTDCSKCSCNPEICKCSTAKKSCCGAK	5:4
	PmiMT6	SRA	MDPCKCAETGSCHCDOCTDCSOCRCNPSNCKCSESGKPCCGSRAK	4:2
	PmiMT7	SRA	MDPCNCAETGTCHCDQCTDCSNCKCNPQNCKCSKPCCPK	4:4
	PmiMT8	SRA	MDPCNCAETGTCHCDQCTDCTKCKCNPETCKCSQAKKSCCPK	6:2
	PmiMT9	SRA	MDPCNCTETGVCKCDOCDCSKCNCDPDTCKCTKASPSGTK	5:2
			MNPCKCAETGSCRCDRCTDCSQCQCNPDNCKCATSGKGCCQGPCKCAEGGPCHCDQCTDCSNCKCAPGNCKCAGSGKECCPRPC	2
			KCAEGGPCHCDECTDCSRCKCAPANCKCAKSGKSCCPEACKCAEGGPCRCDKCTDCSRCKCAPENCKCAKSGKGCCPGPCKCAEGG	i
			PCHCDECTDCSQCKCAPANCKCAKSGKSCCPEPCKCAEGGPCHCNKCTDCSRCKCAPENCKCAKSGKGCCPGPCKCAEGGPCHCD	
	PmiMT10part	SRA	Р	30:10
Polyandrocarpa zorritensis	PzoMT1	SRA	MDPCNCAETGVCKCENCTDCSKCNCNTKTCKCTKSCCPK	6:4
	PzoMT2	SRA	MDPCNCKESGVCHCDKCTDCSKCVCDPDNCKCANVKKTCCPTK	7:3
	PzoMT3	SRA	MDPCNCAETGSCQCQKCNDCSQCKCGSNCKCSPSGKSCCGK	5:3
	PzoMT4	SRA	MDPCNCKETGVCKCDQCSDCSKCNCXPDTCKCTXANKSCCPSGTK	6:3
	PzoMT5	SRA	MDPCKCAEAGTCHCDKCSDCSKCSCNPQNCICTKSCCPK	5:2
	PzoMT6	SRA	MDPCNCKETGVCHCDKCTDCSKCSCXPNTCKCASAKKSCCQK	7:2
	PzoMT7	SRA	MDPCKCAETGTCHCDQCSDCSKCNCNPQNCKCTKSCCPK	5:3
	PzoMT8	SRA	MDPCDCAVTGSCHCQQCKDCKNCKCDPSKCQCAKAGDPAKCLCAKTGTCCCSGKA	8:1
Polycarpa aurata	PauMT1	SRA	MDPCKCAETGSCHCDQCTDCSQCRCNPSNCKCSESGKPCCGSRAK	4:2
	PauMT2	SRA	MDPCNCKETGVCKCDQCDCTKCNCNPDTCKCANAKKSCCPLVRSSRPPMNFFQ	6:5
	PauMT3	SRA	MDPCNCKETGVCVCEKCADCSKCNCNPNTCRCAENHKSCCH	4:5
	PauMT4	SRA	MDPCNCAETGTCHCNQCDDCSKCKCNPDVCKCAKSKKSCCPSK	7:3
	PauMT5	SRA	MDPCNCKETGCCCCDKCTDCSKCNCNTDTCKCANSSKTCCPTK	6:4
	PauMT6	SRA	MDPCNCAETGVCNCNNCTDCSNCKCDPAVCKCSKASKTCCPTK	5:5
	PauMT7	SRA	MDPCNCAETGKCHCNQCDHCSKCKCNPSVCKCAKSSKSCCPSK	7:3
	PauMT8	SRA	MDPCNCAVSGNCHCEQCDDCXGCKCNPDVCKCAKSKKSCCASK	6:3
	PauMT9	SRA	MDPCNCTQTGVCKCDQCQCSKCNCDPDTCKCTKASPSGTK	5:2
	PauMT10nart	SRA		, 35·11
Polycarna mamillaris	PmamiMT1	SRA		7.3
r olycurpu maninaris	PmamiMT2nar	5101		7.5
	t	SRA	MDPCNCAETGNCHCNQC	0:3
			MNPCKCAETGSCRCDRCTDCSQCRCNPDNCKCSTSGKGCCQGPCKCAEGGPCHCDQCTDCSNCKCAPGNCKCAGSGKECCRRPC	
			KCAEGGPCHCDKCTDCSRCECAPANCKCAKSGKSCCPEACKCADGGPCRCDKCTDCSQCKCAPENCKCAKSGKGCCPGPCKCAEG	
			GPCHCNECTDCSQCKCAPANCKCSESGKSCCPGPCKCAEGGPCHCNKCTDCSRCECAPENCKCAKSGKGCCPVPCKCAEGGPCHC	
			NKCTDCSOCKCAPENCKCAKSGKGCCPGPCKCAEGGPCHCDOCTDCSOCKCAPGNCKCATSGKACCGRPCECADTGFCRCNKCTD	)
	PmamiMT3	SRA	CSQCRCNPKNCKCATAGKRCCRGTA	41:17
Polycarpa pomaria	PpoMT1	SRA	MDPCNCKETGVCKCDQCSDCSKCNCXPDTCKCTXANKSCCPSGTK	6:3
, , , ,	PpoMT2part	SRA	DCSKCNCNPONCKCTKSCCPK	4:3
	PpoMT3	SRA	MDPCNCKETGVCHCDKCTDCSKCSCDPNTCKCASAKKSCCOK	7:2
	PpoMT4	SRA		8.7
	PpoMT5	SRA		6.3
	PpoMT6	SRA	MDPCNCKFSGVCHCDKCKCDCDNCKCANVKKTCCPTK	7.3
	, pointe		MNPCKCAFTGSCOCDRCTDCSOCRCNPDNCKCATSGKGCCOGPCKCAFGGPCHCDOCTDCSNCKCAPGNCKCAGSGKECCPRPC	
			KCAEGGPCHCDECTDCSKCECAPANCKCAKSGKSCCPEACKCAFGGPCRCDKCTDCSRCKCAPENCKCAKSGKGCCPGPCKCAFGG	-
			PCHCNECTDCNOCOCAPANCKCAKSGKSCCPGPCKCAEGGPCYCNKCTDCSOCKCAPENCKCSKAGKGCCPGPCKCAEGGPCHC	
			NKCTDCSQCKCAPENCKCAKSGKGCCPGPCKCAEGGPCHCDOCTDCSQCKCAPGNCKCATSGKACCGPCACADTGSCRCDKCTD	
	PpoMT7	SRA	CSOCRCNPKNCRCATAGKOCCRGKA	42.17
Polycarna sn	PsnMT1	SRA	MDPCNCAFTGTCHCDOCTDCTKCKCNPFTCKCSOAKKSCCPK	6.2
· •···•	PsnMT2	SRA	MDPCNCKVTGOCHCDOCDDCSKCNCDPAVCKCTKSCCPK	5.2
	. op			J

			PspMT3	SRA	MDPCNCTETGVCKCDQC-DCSKCNCDPDTCKCTKASPSGTK	5:2
			PspMT4	SRA	MDPCNCAVSGNCHCEQCDDCXGCKCNPDVCKCAKSKKSCCASK	6:3
			PspMT5	SRA	MDPCNCKETGVCKCDQCSDCTKCNCNPDTCKCANAKKSCCPSGTK	7:4
			PspMT6	SRA	MDPCNCAETGVCNCNNCTDCSNCKCDPAVCKCSKASKTCCPTX	4:5
					MNPCKCAETGSCRCDRCTDCSQCQCNPDNCKCATSGKGCCQGPCKCAEGGPCHCDQCTDCSNCKCAPGNCKCAGSGKECCPRPC	С
					KCAEGGPCHCDECTDCSRCKCAPANCKCAKSGKSCCPEACKCAEGGPCRCDKCTDCSRCKCAPENCKCAKSGKGCCPGPCKCAEGG	ŝ
			PspMT7part	SRA	PCHCDECTDCSQCKCAPANCKCAKSGKSCCPEPCKCAEGGPCHCNKCTDCSRCKCAP	26:9
		Stolonica socialis	SsoMT1	SRA	MDPCNCAVSGNCHCEQCDDCXGCKCNPDVCKCAKSKKSCCASK	6:3
			SsoMT2	SRA	MDPCNCKVTGQCHCDQCDDCSKCNCDPAVCKCTKSCCPK	5:2
			SsoMT3	SRA	MDPCNCAETGTCHCDQCTDCTKCKCNPETCKCSQAKKSCCPK	6:2
			SsoMT4	SRA	MDPCNCAETGVCNCNNCTDCSNCKCDPAVCKCSKASKTCCPTK	5:5
			SsoMT5	SRA	MDPCNCKETGVCHCXQCTDCSKCNCNPNTCKCSSAKKSCCSK	6:4
			SsoMT6	SRA	MDPCNCTETGVCKCDQCDCSKCNCDPDTCKCTKASPSGTK	5:2
			SsoMT7	SRA	MDSCNCKDTGRCHCNKCTDCSNCKCNPNTCKCAKSNCCPK	6:6
					MNPCKCAETGSCRCDRCTDCSQCQCNPDNCKCAXSGKGCCQGPCKCAEGGPCHCDQCTDCSNCKCAPGNCKCAGSGKECCPRP	
					CKCAEGGPCHCDECTDCSRCKCAPANCKCAKSGKSCCPEACKCAEGGPCRCDKCTDCSRCKCAPENCKCAKSGKGCCPGPCKCAEG	i
			SsoMT8part	SRA	XPCHCDQCTDCSQCKCAPGNCKCATSGKACCGRPCACADTGSCRCDKCTDCSRCRCNPENCKCA	24:10
		Styela canopus	ScaMT1	SRA	MDPCNCKETGVCKCDQCSDCSKCNCSPDTCKCTEANKSCCPSGTK	6:3
			ScaMT2	SRA	MDPCKCAEAGTCHCDKCSDCSKCSCNPQNCICT	3:2
			ScaMT3	SRA	MDPCKCAETGTCHCDQCSDCSKCNCNPQNCKCTKSCCPK	5:3
			ScaMT4	SRA	MDPCNCKETGVCHCDKCTDCSKCSCDPNTCKCASAKKSCCQK	7:2
			ScaMT5	SRA	MDPCNCAETGVCNCTQHADCGKCSCNPEVCKCSQAKKGCCGAK DKCTDCSKCNCNPEACKCASGCCGSATVRPCNCKETGTCHCDKCTDCSNCKCNPEMCKCTTGCCANTTVGPCNCKETGTCHCDKC	5:3
			ScaMT6part	SRA	TDC	9:7
		Styela clava	ScIMT1	SRA	MDPCNCAETGTCHCDKCTDCTKCKCNPETCKCSQAKKSCCPK	7:2
			ScIMT2	SRA	MDSCNCKDTGRCHCNKCTDCSNCKCNPNTCKCAKSNCCPK	6:6
			ScIMT3	SRA	MDPCNCKETGVCVCENCADCSKCNCNPNTCRCAENHKSCCH	3:6
			ScIMT4	SRA	MDPCNCKETGVCHCDKCTDCXKCNCNPETCKCQKSCCPK	6:3
			ScIMT5	SRA	MDPCNCKVTGQCHCDQCXDCSKCNCDPAVCKCTKSCCPK	5:2
			ScIMT6	SRA	MDPCKCAETGSCHCDQCTDCSQCRCNPSNCKCSESGRACCGSQAK	3:2
			ScIMT7	SRA	MDPCNCAETGVCNCNNCTDCSNCKCDPAVCKCSKASKTCCPTK	5:5
			ScIMT8	SRA	MDPCNCXETGVCKCDQCSDCTKCNCNPDTCKCANAKKSCCPSGTK	6:4
			ScIMT9	SRA	MDPCNCTETGVCKCDQCDCSKCNCDPDTCKCTKASPSGTK	5:2
		Styela gibbsii	SgiMT1	SRA	MDPCNCAETGXCNCQNCTDCSKCNCNPETCKCAQSKKSCCPSK	5:5
			SgiMT2	SRA	MDPCNCAETGNCTCNPNSCDCSKCNCNPETCKCTKAGKSCCDKTK	6:6
		Styela plicata	SpIMT1	SRA	MDPCNCKETGNCKCDTCTDCSKCNCNPSTCKCKACKKTCCG	7:4
			SpIMT2	SRA	MDPCNCAVTGKCHCPECTDCSKCTCKPETCKCAQARKSCCPSK	6:1
			SpIMT3	SRA	MDPCNCAETGRCACDPTNCKCSECNCHPDTSKCKKAEKSCCEKK	7:3
Pyrosomatida	Pyrosomatidae	Pyrosomella verticillata	PveMT1	SRA	MDPCNCKVTGKCHCDQCTDCSKCNCASPCKCTKSCC	5:2
			PveMT2	SRA	MDPCNCKITGNCHCAQCTDCSNCSCAAPCKCTKSCC	3:3
Salpida	Salpidae	lasis cylindrica	IcyMT1	SRA	MDPCNCETTRDCHCQXCTDCSRCKCDLKNCNCSKPCCPK	4:3
			IcyMT2	SRA	MAYCNCLSNSNCHCNTCTDCSKCACAKNTCSCSGKTCCSPQ	3:5
		Salpa fusiformis	SfuMT1	SRA	MDPCNCNTSDMCHCESCNDCSKCNCARQTCKCSTKRCCSPK	4:4
			SfuMT2	SRA	MDPCNCQVTGVCHCNQCDDCSKCSCKPSNCKCNKPCCPK	5:4
			StuMT3	SKA	MDPUNUUDIUSCYCSSCTDCSKCTCAKTTCKCSAKGCCSPN	4:2
		o 1 – T	SfuMT4	SRA	MDSCNCKGTQSCHCNTCQDCSKCTCDKRTCKCSGKGCCSPE	5:2
		Salpa Thompsoni	SthMT1	MW558888	MDPUNUNISDMCHCDSCKDCSKCNCARKTCKCSTKGCCSPK	6:3
			SthMT2	MKHR01072568	MDPCNCKVTGACHCDQCTDCGKCSCNPANCKCSKPCCPK	5:3
			SthMT3	MKHR01021011		4:2
			StnMT4part	WIKHR01216936		3:3
			SthMT5	MW558889	MDPCNUNTSDMCHCESCKDCSKCNCAKQTCKCSTKRCCSSK	6:3

Thaliaceans

				SthMT6	MW558890	MDPCNCKVTGACHCDQCTDCSKCNCNPANCKCSKPCCPK	5:4
Larvacear	IS						
	Copelata	Oikopleuridae	Oikopleura albicans	OalMT1part	sc7	SNNDGKSVCSGCKNCPPGCNPCKCPKCSEHGCDCSCCNDKPCLARCVGCANCPPDCNPCYCNKCRTNGCSCACCPDDKPCVSRC GCKNCPPDC MSEPDTDADSDCCGRQCDQCTNCPPNCDPCGCTKCNQRGCSCSCCDDKKKCCDCCQEDCNCTDCESCDSGYCKNSCGCRKNNI FRKGCCNEGDSCWESNDPPGCNPCKCPKCSKNGCDCSCCDDKPCLPRCDNCANCPPDFCACCPDDKPCVSRCDGCKNCPPDCKF	.D 8:10 LC PC
				OalMT2part	sc8914	KCLKCSANGCTCGCCEKVNNCCDC CTKCSQKGCSCCCDDQKTGCECCKDCDCCKDDCNCTDCESCDCGCCKSNACCDTDCCESKNKCCDCCKDCDGCKDCSAEGCGP TCDCCQTNSCCEQGCCNVEKKKCCDCCKDCDCCDGCGGNSCGPCSCGCCKNNLCCKKGCCNEGDSCCDGNTEGDSCCGSTSEC PVCSGCKNCPPGCNPCKCPKCSKNGCDCSCCDDKPCVARCDGCANCPPDCKKCKTNGCSCACCPPDKPCVSRCDGCKNC PDCPCKGACKACCCCCAV/SNCCDCCCDCCDCCDCCDCCDACC/CVANVCCCCCCAV/CVCCAPKCCCDCCACCICCCCCCA/	17:17 YC K CP
				OalMT3part	sc11769		40:26
			Oikopleura dioica	OdiM11	AYN64376.1	MDPVCSFRCCEENCAGCVDCPAGCDPCKCTLEVCKKVCEGCKDCPPGCEPCKCEKCSTKKCKSNCCPTSTAE	9:2
				OdiMT2	AYN64373.1	MEVKRPNNCCPAKCLGCKGCPPGCEPCICNMDTCKNICNKCKECPKNEFGCDPCKCPKCSKLGCTCDCCHKKCCVTDCDGCKTCP GCEPCKCSMNACKKVCKQCKNCRKSESGCDPCECSKCALKGCKCDCCPKDTCCEASCEGCKNCPPGCEPCKCTLNCCMKICDDCK CPKSENGCDPCNCRKCSRKGCNCDCCPSDDCCKASCEGCINCPPGCDPCECSMDECKKICKKCNNCRKGESGCDPCECRKCSRNG DCDCCPKDSCCEASCEGCTDCPQGCKPCKCTMNSCMKTCDKCKDCPKSASGCDPCECLKCSRKGCECDCCPQKNDCCEAFCQGC NCPPGCNPCKCTLNFCAKICNECKDCPKSDIGCDPCNCVKCSAKGCKCDCCPKKCC	'P D iC :K 57:24
						MNPLAEKSKCATKCAGCKDCPLNCDPCKCCGCSCCGAGGLKLCRSQICEGCRNCPPGCDPCLCPKCKNSGCSCACCEKKPSCNKIC GCANCPPGCDPCLCAKCHNNGCTCSCCADKPCMVRCAGCANCPRNCEPCLCSKCSKNGCDCSCCAKNPCKARCAGCADCPPNC	:A :D
			Oikopleura vanhoeffeni	OvaMT1	sc728	PCLCSKCSKNGCDCSCCEKKPCASICDGCKDCPPGCKPCKCADCRKNGCGCSCCTTQSNENECC CKKCPINCEPCMCOICNONGCNCACCAEKPCMVRCAGCAKCPPNCEPCI CSKCNTKGCNCSCCANKPCKVKCAGCANCPANCDI	25:17 P
				OvaMT2part	sc8397	CFCPKCSKIGCNCSCCENKPCPSICDKCKYCPPGCEPCRCADCRKNGCNCSC	15:14
				OvaMT3part	sc62250	CPPNCDPCFCPKCSKNGCGCSCCENKPCASICDGCKDCPPGCKPCKCADCRKNGCNCSC	7:5
						AKPCCGQICEGCKNCPPGCDPCLCPKCKKSGCSCCCEKQPCCGPICEGCKNCPPGCDPCLCPKCKKSGCSCACCDKKPSCNKVCAC	G
				OvaMT4part	sc79129	CANCPPGCNPCLCPQCSKNGCTCSCCVDKPCNVRCKGCANCPPNCEPCLCPKCSTNGCSCCANKPCKVRCAGCAN	19:12

<sup>1</sup>C. robusta formerly C. intestinalis type A <sup>2</sup>C. intestinalis formerly C. intestinalis type B \* EST sequences

	SthMT1	SthMT2	SthMT3	SthMT4_part	SthMT5	SthMT6	
SthMT1		60,98%	68,29%	66,67%	87,80%	65,85%	
SthMT2	65,85%		58,54%	42,42%	56,10%	94,87%	_
SthMT3	78,05%	70,73%		52,52%	65,85%	60,98%	den
SthMT4_part	84,85%	54,55%	66,67%		69,70%	48,48%	ıtity
SthMT5	95,12%	63,41%	70,73%	84,85%		60,98%	
SthMT6	68,29%	97,44%	70,73%	57,58%	65,85%		
			Sin	nilarity			

Table S2. Percentatge of Similarity (down) and identity (up) in protein sequence obtained with GENEIOUS prime (Geneious Prime® 2020.1.2)

TABLE S3. Number of Identical bases in front of the total alignment and percentage of Identity between SthMT1/5 and SthMT2/6.

	5' UTR		INTRON		3' UTR	
	Identity number/Total alignment	% identity	Identity number/Total alignment	% identity	Identity number/Total alignment	% identity
SthMT1 vs SthMT5	41/45	91,11%	339/389	87,14%	186/225	82,66%
SthMT2 vs SthMT6	28/28	100,00%	323/395	81,77%	61/71	85,91%

Table S4: PCR amplification primers for Salpa gDNA.

	Primer	Sequence	T <sup>a</sup> Annealing	
SthMT1	FwMT1/MT5	CGTCATACTTTTGTGCTTCAAAC	66	
	RvMT1/MT2	TACACKRATCRCAATGRCA	55	
SthMT5	FwMT1/MT5	CGTCATACTTTTGTGCTTCAAAC	FO	
	RvMT5	AATCAATTCACAATTGCAAATC	50	
SthMT6	FwMT2/MT6	CTATCGAACGAATCACTTCAAC	E4	
	RvMT6	GGAAAAAGACAAGACAATTC	54	