Evolutionary History of the Globin Gene Family in Annelids

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ABSTRACT

Animals depend on the sequential oxidation of organic molecules to survive; thus, oxygen-carrying/transporting proteins play a fundamental role in aerobic metabolism. Globins are the most common and widespread group of respiratory proteins. They can be divided into three types: circulating intracellular, non-circulating intracellular, and extracellular, all of which have been reported in annelids. The diversity of oxygen transport proteins has been underestimated across metazoans. We probed 250 annelid transcriptomes in search of globin diversity in order to elucidate the evolutionary history of this gene family within this phylum. We report two new globin types in annelids, namely androglobins and cytoglobins. Although cytoglobins and myoglobins from vertebrates and invertebrates are referred to by the same name, our data show they are not genuine orthologs. Our phylogenetic analyses show that extracellular globins from annelids are more closely related to extracellular globins from other metazoans than to the intracellular globins of annelids. Broadly, our findings indicate that multiple gene duplication and neo-functionalization events shaped the evolutionary history of the globin family.

Keywords: androglobin, cytoglobin, extracellular globin, gene tree, transcriptomics, respiratory proteins

List of abbreviations: Adgb, androglobin; Cygb, cytoglobin; Hb, hemoglobin; HBL-Hb, hexagonal bilayer hemoglobin; His, histidine; Mb, myoglobin; MW, molecular weight; nHb, nerve hemoglobin; O$_2$, oxygen; Phe, phenylalanine; RBC, red blood cell.

Significance Statement: Annelid worms have the greatest diversity of oxygen-carrying proteins, also known as blood pigments, among all animals. However, the real diversity of these proteins has been still underestimated. To access the diversity of globins present in annelids, and to elucidate their evolutionary relationships, we have searched for globin genes in 250 annelid genetic material. We found two new globins in this phylum: androglobins and cytoglobins. Our results indicate that cytoglobins and myoglobins from vertebrates and invertebrates have different evolutionary origins, and that androglobins and extracellular globins originated early in animal’s evolution. We show that multiple gene duplication events shaped the complex evolutionary history of the globin famil

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INTRODUCTION

Aerobic metabolism relies on the sustained transfer of oxygen (O$_2$) from environmental sources to the respiring tissues of animals, which is carried out by O$_2$ transport proteins (also known as respiratory pigments) (Terwilliger, 1998; Burmester and Hankeln, 2004; Coates and Decker, 2017). These globular proteins represent the most widespread respiratory pigments and occur almost ubiquitously amongst organisms, including bacteria, fungi, plants, protists, and animals (Hardison, 1996, 1998; Weber and Vinogradov, 2001; Vazquez-Limon et al., 2012; Vinogradov et al., 2007, 2013a, b). Concerning metazoans, intra- and extra-cellular hemoglobins (Hb and HBL-Hb, respectively) and myoglobin (Mb) have been known for over a century (Lankester, 1872). More recent comparative genomic studies revealed the existence of several new globin types in vertebrates, such as cytoglobin (Cygb), androglobin (Adgb), and neuroglobins (Ngb) (Burmester et al., 2000; Kawada et al., 2001; Burmester et al., 2002; Trent and Hargrove, 2002; Burmester and Hankeln, 2004; Hoogewijs et al., 2012). Following this trend, studies demonstrated that the known diversity of oxygen-carrying proteins in animals is underestimated, and this also seems to be true in annelids (Bailly et al., 2008; Martín-Durán et al., 2013; Costa-Paiva et al., 2017, 2018; Belato et al., 2019). Despite the conserved tertiary structures of all globins, the recently discovered proteins differ markedly in their amino acid sequences and carry out several alternative cellular functions besides O$_2$ transport, e.g., oxygen-sensing, enzymic activity, signal transduction, lipid and nitric oxide metabolism, and detoxification of reactive oxygen species, suggesting that the presence of more than one type of oxygen-binding protein in animals is related to those other cellular functions (Weber and Vinogradov, 2001; Burmester and Hankeln, 2014).

The circulating annelid Hbs occur within nucleated red blood cells (RBCs), in contrast to the anucleate RBCs that harbor the intensively studied mammalian Hbs (Storz, 2018). Invertebrate Hbs are found in at least six phyla: Phoronida, Nemertea, Mollusca, Annelida, Arthropoda, and Echinodermata (Terwilliger and Ryan, 2001; Weber and Vinogradov, 2001), where they may occur in closed vascular systems, or dissolved in the coelomic fluid and hemolymph (functional equivalents to blood). Annelid Hbs exhibit the classical “Mb-fold”, a structure that comprises five to eight α-helices, named A through H, forming a three-on-three or two-on-two helical sandwich that surrounds the oxygen-binding heme group (Bolognesi et al., 1997; Terwilliger, 1998; Weber and Vinogradov, 2001; Vinogradov and Moens, 2008; Gell, 2018). All invertebrate Hbs contain the characteristic, invariant globin amino acids residues:
His at E7 (seventh amino acid in helix E), His at F8, and Phe at the inter-helical region CD1 (Bolognesi et al., 1997; Weber and Vinogradov, 2001). In contrast to the tetrameric vertebrate Hbs, annelid RBC Hbs may be monomeric, dimeric, tetrameric, polymeric or a combination of these states (Weber, 1980; Mangum, 1985). The hexagonal bilayer hemoglobins (HBL-Hbs), also called chlorocruorins and erythrocruorins, are giant (MW ~3.5x10^6), extracellular circulating protein complexes that occur freely dissolved in blood equivalents (Weber, 1971; Weber and Vinogradov, 2001). For decades, the HBL-Hbs were considered to be present only in a few annelid species (Vinogradov, 1985; Weber and Vinogradov, 2001). Recently, we demonstrated a much wider phylogenetic distribution of these giant extracellular proteins in invertebrates, including Mollusca, Platyhelminthes, and some deuterostome lineages (Belato et al., 2019). The mega-molecular HBL-Hbs are comprised of two types of polypeptides: globin chains, with single oxygen-binding sites that satisfy the criterion of a globin-like fold, and linker chains that lack heme groups and are required for the multimeric (hierarchical) assembly of the vast quaternary structures (Vinogradov, 1985; Lamy et al., 1996; Weber and Vinogradov, 2001; Royer et al., 2006).

Among the intracellular non-circulating globins, myoglobins (Mbs) are monomers consisting of ~140 amino acids that reside in the cytoplasm of muscle cells of metazoan taxa and function as intracellular O\textsubscript{2} store and in transcellular (facilitated) diffusion of O\textsubscript{2} (Wittenberg, 1970; Suzuki and Imai, 1998). Non-circulating globins also comprise the nerve hemoglobins (nHbs), that occur sporadically in glial cells surrounding the nerve cord and neurons of various invertebrate taxa, including Annelida, Arthropoda, Echiura, Mollusca, Nematoda, and Nemertea (Wittenberg, 1992, Weber and Vinogradov, 2001; Geuens et al., 2004; Burmester and Hankeln, 2008). nHbs consist of ~150 amino acid residues and may exhibit the Mb-like structure and exist as homodimers, as seen in the annelid *Aphrodita aculeate* (Wittenberg, 1992; Dewilde et al., 1996; Weber and Vinogradov, 2001; Geuens et al., 2004). Although all nHbs contain the diagnostic residues (Phe CDI, HisE7, and HisF8), phylogenetic analyses indicate divergent evolutionary origins (Wittenberg, 1992; Dewilde et al., 1996; Weber and Vinogradov, 2001; Burmester and Hankeln, 2008). The principal function of invertebrate nHbs is considered to be O\textsubscript{2} storage and supply during hypoxia, sustaining the aerobic metabolism of the nervous system (Kraus et al., 1988; Wittenberg, 1992, Weber and Vinogradov, 2001; Geuens et al., 2004).

Cytoglobins (Cygbs) are non-circulating globins that are co-located alongside Mbs in the cytoplasm of cells of several different vertebrate tissues. However, Cygbs have longer polypeptide chains, with around 170 amino acid residues, since additional residues flank the N- and C- terminals and they
thus lack sequence insertions that interrupt the globin fold (Burmester et al., 2002). These proteins do not contain signal peptides and are found in the cytoplasm and nucleus of many different cell types (Burmester et al., 2002). Vertebrate Cygb shows structural and phylogenetic affinities to vertebrate Mb (Burmester et al., 2002; DeSanctis et al., 2004); however, these relationships are not resolved for invertebrate Cygbs. Androglobins (Adgbs), the most recently discovered non-circulating globins, are cytoplasmic, large chimeric proteins that exhibit a modular domain structure. They comprise a N-terminal calpain-like domain, a rearranged globin domain, where the eight α-helices (A-H) are organized such that helices C-H precede helices A-B, and an IQ calmodulin-binding motif (Hoogewijs et al., 2012; Bracke et al., 2018). Despite the different globin domain sequence, Adgbs satisfy the globin-fold criterion (Hoogewijs et al., 2012; Bracke et al., 2018). These chimeric proteins have been recorded in a wide range of metazoan taxa, such as Mollusca, Cnidaria, and Chordata (Hoogewijs et al., 2012; Bracke et al., 2018).

Annelids thus exhibit the greatest diversity of oxygen-binding proteins among metazoans (Mangum, 1998; Costa-Paiva et al., 2017), with three types of globins described so far: 1) non-circulating cytoplasmic globins such as Mbs and nHbs; 2) circulating intracellular red blood cell (RBC) Hbs and 3) extracellular HBL-Hbs dissolved in body fluids (Wittenberg, 1970; Vinogradov et al., 1993; Lamy et al., 1996; Suzuki and Imai, 1998; Weber and Vinogradov, 2001; Bailly et al., 2007). Related to the scarcity of available sequences for these three globin types in annelids, only three families are known to express all simultaneously: Opheliidae, Terebellidae, and Alvinellidae (Weber, 1978; Hourdez et al., 2000). To the best of our knowledge, only one study (Bailly et al., 2007) has focused exclusively on the evolutionary history of the globin superfamily in annelids. Using 28 annelid globin sequences, Bailly et al. (2007) demonstrated that extracellular globin lineages appear to have a separate evolutionary history compared to intracellular circulating and noncirculating annelid globins. Nevertheless, the real diversity of globin genes within annelids is yet to be investigated, and phylogenetic relationships between different globin types in these animals remain uncertain. In order to access the real diversity of globins present in annelids, and to elucidate the evolutionary relationships of these proteins within the phylum, our work represents a systematic analysis of 250 annelid transcriptomes to survey for globin genes. We report the existence of four non-circulating intracellular globin types in annelids: Mbs, nHbs, Cygbs and Adgbs. Our molecular evolutionary analyses indicate a complex evolutionary history for members of the globin superfamily within Annelida, which includes several gene duplication and neo-functionalization events.

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METHODS

Transcriptomes of 250 annelid species were used in this work and information about each species is indexed in Supplementary file 1. The transcriptomes were collected as part of the WormNet II project that primarily seeks to resolve annelid phylogeny. Specimens were obtained by several collection techniques, including intertidal sampling, dredging, and box cores. Afterwards, all samples were preserved either in RNALater or frozen at −80 °C. Protocols from Kocot et al. (2011) and Whelan et al. (2015) were used for RNA extraction, cDNA preparation and high-throughput sequencing. Succinctly, total RNA was extracted using TRIzol (Invitrogen) either from whole small animals, or from the body walls and coelomic regions, in bigger specimens. After extraction, RNAs were purified using the RNeasy kit (Qiagen) with on-column DNase digestion. To reverse transcribe single-stranded RNA template, the SMART cDNA Library Construction Kit (Clontech) was used and double-stranded cDNA synthesis was performed with the Advantage 2 PCR system (Clontech). The Genomic Services Lab at the Hudson Alpha Institute (Huntsville, AL) was responsible for barcoding and sequencing libraries with Illumina technology. Considering that transcriptomic sequencing was conducted from 2012 to 2015, paired-end runs were of 100 or 125 bp in length, utilizing either v3 or v4 chemistry on Illumina HiSeq 2000 or 2500 platforms (San Diego, CA). Finally, in order to facilitate sequence assembly, paired-end transcriptome data were digitally normalized to an average k-mer coverage of 30 using the script normalize-by-median.py (Brown et al., 2012) and was assembled using Trinity r2013-02-25 with default settings (Grabherr et al., 2011).

Bioinformatic methods employed to search in silico for genes of the globin family were similar to those in Belato et al. (2019). Transcriptome data was processed through the Trinotate annotation pipeline (http://trinotate.github.io/) (Grabherr et al., 2011). The Trinotate pipeline uses a BLAST-based method against two databases, namely EggNOG 4.5.1 (Huerta-Cepas et al., 2016) and KEGG (Kanehisa et al., 2016), to provide the Gene Ontology (GO) annotation. The GO is a standardized functional classification system for genes that describes the properties of genes and their products using a dynamic-updated controlled vocabulary (Gene Ontology Consortium, 2004). The complete list of software employed by the Trinotate pipeline to provide the annotation of genes is: HMMER 3.2.1, for protein domain identification (Finn et al., 2011); tmHMM 2.0, for prediction of transmembrane helices of proteins (Krogh et al., 2001); RNAmmer 1.2, for prediction of ribosomal RNA (Lagesen et al., 2007);
SignalP 4.1, to predict signal peptide cleavage sites (Petersen et al., 2011); GOseq, for prediction of the gene ontology (Young et al., 2010); and EggNOG 4.5.1, for searching orthologous groups of genes (Huerta-Cepas et al., 2016). As we used transcriptomic data, we can only make inferences about the presence of gene signatures and refrain from drawing conclusions about their absence, since genes may be present in the genome without being expressed in the sampled tissue at time of collection.

Retrieved sequences were manually verified by inspecting each functional annotation made by Trinotate in order to select sequences annotated as hemoglobins (Hbs), myoglobins (Mbs), cytoglobins (Cygbs), androglobins (Adgbs), and nerve hemoglobins (nHbs). In addition, 10 annelid extracellular hemoglobin (HBL-Hbs) sequences from Belato et al. (2019) were selected to be used in our analyses because these sequences were obtained employing the same bioinformatic pipeline including the same annotation and validation steps. RNA sequences identified as one of the genes described above were then translated into amino acids using TransDecoder software with default settings (https://transdecoder.github.io/). All translated protein sequences were evaluated using the Pfam domain check (Finn et al., 2016) employing the EMBL-EBI protein database with an e-value cutoff of $10^{-5}$. This step was necessary because the TransDecoder translation may produce multiple open reading frames (ORFs). Translations returning with a confirmed Pfam domain and that were longer than 130 amino acids residues were retained for further analysis. In order to refine the results, we added a confirmatory step, where we performed a reciprocal BLASTp (Altschul et al., 1990) of all sequences annotated as the target genes against the non-redundant protein database (nr) from the National Center for Biotechnology (NCBI). Only sequences that presented a significant top ‘hit’ with a minimum e-value of $10^{-10}$ to one of the target genes Hbs, Mbs, Cygbs, Adgbs, nHbs and HBL-Hbs were retained. We have labeled proteins according to their putative functional role, considering local similarity between sequences from our dataset and NCBI database. Sequence similarity and similarity in domain structure are generally indicative of similarity in function (Marcotte et al., 1999; Ashburner et al., 2000; Gabaldón and Huynen 2004).

Adgbs were manually rearranged in order to remove the IQ motif and concatenate the eight $\alpha$-helices (A-H) of the globin domain that are inverted in these globins. After all validation steps, the remaining 379 sequences were aligned with MAFFT using the accurate algorithm E-INS-i (Katoh and Standley, 2013), and gap-rich regions in the alignment were removed with trimAl 1.2 (Capella-Gutierrez et al., 2009) using a gap threshold of 0.75. The alignment was manually curated using the software http://mc.manuscriptcentral.com/gbe
Geneious 11.1.2 (Kearse \textit{et al.}, 2012) in order to remove spuriously aligned sequences based on similarity to the protein alignment as a whole. To eliminate dataset redundancy sequences that presented 100\% of similarity to each other were also excluded from the alignment. The resulting amino acid alignment of 238 sequences was subsequently used for phylogenetic analyses.

ModelFinder, an ultrafast and automatic model selector implemented in IQ-TREE software (Kalyaanamoory \textit{et al.}, 2017) was applied to carry out statistical selection of the best-fit model of protein evolution for the dataset using the Akaike and Bayesian Information Criteria (AIC and BIC, respectively) (Darriba \textit{et al.}, 2011). Two phylogenetic inference methods were employed: (a) a maximum likelihood inference performed with the IQ-TREE software (Nguyen \textit{et al.}, 2015) with branch support obtained with the ultrafast bootstrap approximation (UFBoot) with 1,000 replicates (Minh \textit{et al.}, 2013) and (b) a Bayesian inference using MrBayes 3.2.7 (Ronquist and Huelsenbeck, 2003) with two independent runs, each one containing four Metropolis-coupled chains that were run for $10^7$ generations and sampled every 500th generation to approximate posterior distributions. To confirm whether chains achieved stationary and to determine an appropriate burn-in, we evaluated trace plots of all MrBayes parameter outputs in Tracer v1.6 (Rambaut \textit{et al.}, 2014). The first 25\% of samples were discarded as burn-in and a majority rule consensus tree was generated using MrBayes. Bayesian posterior probabilities were used for assessing statistical support of each bipartition. The resultant trees were summarized with FigTree 1.4.3 (Rambaut, 2009) and rooted by midpoint rooting (Farris, 1972; Hess and Russo, 2007). The Phyre2 web portal (Kelley \textit{et al.}, 2015) was used to predict the putative tertiary structures of the different globins and models were visualized and inspected using UCSF Chimera (Pettersen \textit{et al.}, 2004).

In order to better understand the evolutionary relationship between extracellular globins of annelids and those from other metazoans, another maximum likelihood analysis was performed using the IQ-TREE software (Nguyen \textit{et al.}, 2015) with branch support obtained under the ultrafast bootstrap approximation (UFBoot) (Minh \textit{et al.}, 2013). This analysis expanded the original 238 sequences alignment with another 15 extracellular globin sequences from five metazoan species obtained from Belato \textit{et al.} (2019): \textit{Astrotoma agassizii} (Echinodermata; MH995909 and MH996362), \textit{Cephalodiscus gracilis} (Hemichordata; MH995925–26), \textit{Hemithiris psittacea} (Brachiopoda; MH996374–75 and MH996036–38), \textit{Phoronis psammophila} (Phoronida; MH996210–11), and \textit{Priapulus} sp. (Priapulida; MH996240–42 and MH996405).
To access the evolutionary relationships between annelid globins and other metazoan globins, we selected a representative panel of 54 globins from deuterostomes (including vertebrates) and other protostomes from NCBI to be used as references of metazoan globins (Supplementary file 2). Together with a subset of 43 annelid globins (Table 1), these 97 sequences of annelid globins + metazoan globins were aligned with MAFFT using the accurate algorithm E-INS-i (Katoh and Standley, 2013), and gap-rich regions in the alignment were removed with trimAl 1.2 (Capella-Gutierrez et al., 2009) using a gap threshold of 0.50 (Supplementary file 3). Afterwards, a maximum likelihood analysis was performed using the IQ-TREE software (Nguyen et al., 2015) with branch support obtained under the ultrafast bootstrap approximation (UFBoot) (Minh et al., 2013).

RESULTS

The initial Trinotate analysis recovered 5,267 nucleotide sequences annotated as Hbs, Mbs, Cygbs, Adgbs, nHbs or HBL-Hbs. After all processing steps, including translation from nucleotides to amino acids, selection by minimum size, reciprocal BLASTp, and Pfam domain evaluation, our in-silico analyses recovered 238 unique amino acid sequences. These sequences consisted of 130 sequences of Hbs, 19 sequences of Mbs, 27 sequences of Cygbs, four sequences of Adgbs, 39 sequences of nHbs and 19 sequences of HBL-Hbs genes (Table 1). These genes are actively transcribed in 121 annelid species belonging to 64 different families as detailed in Table 1. Accession numbers of each one of the sequences obtained in this work were deposited in GenBank and are listed in Table 1 and detailed in Supplementary file 4.

The number of expressed Hbs genes in a given species ranged from one in 48 different species to eight in Terebellides stroemii (Trichobranchidae). For Mbs, the number of expressed genes in a given species ranged from one in 10 different species to four in Praxillella pacifica (Maldanidae). For Cygbs the corresponding numbers were one in 25 different species and two in Aglaophamus verrilli (Nephtyidae). One Adgb gene was found in four different species, and nHbs genes ranged from one expressed copy in 24 different species to three in Alciopa sp. (Alciopidae). Besides the 10 HBL-Hbs sequences selected from Belato et al.’s (2019) previous work that were used as reference (GenBank accession numbers: MH995867–68, MH995870–71, MH995874, MH995876, MH995879–80, MH996356 and MH995881), we found more HBL-Hb genes, ranging from one in two different species to
three in *Heterodrilus* sp. (Naididae). Besides the two well-known intracellular non-circulating globin types found in annelids, Mbs and nHbs, we reveal the presence of cytoglobins (Cygbs) and androglobins (Adgbs) as two new members of this globin type. Additionally, we found three more families that express all globin types (extracellular circulating, intracellular circulating and intracellular noncirculating) simultaneously: 1) Aeolosomatidae (*Aeolosoma* sp.); 2) Arenicolidae (*Abarenicola pacifica*); and 3) Nephtyidae (*Aglaophamus verrilli*).

Following trimming and alignment of translated transcripts the final alignment had the maximum sequence length of 142 residue positions (Supplementary file 5). All sequences in the alignment contained the essential residues of the globin domain (Phe at CD1, His at E7, His at F8), which is an indicator of respiratory function for each one of these proteins (Lecomte et al., 2005; Fig. 1). The best-fixed rate model for phylogenetic analyses of the annelid globins dataset and the annelid globins + metazoan extracellular globins dataset was the WAG model. For phylogenetic analyses of the annelid globins + metazoan globins dataset the best-fixed rate model was LG.

Bayesian and maximum likelihood inferences recovered the same topology with several strongly supported clades in the annelid globins tree (Fig. 2), although the topology did not mirror the recent Annelida phylogeny (e.g., Struck et al., 2015; Weigert and Bleidorn, 2016). Adgb genes clustered into a highly supported monophyletic group by bootstrap values and posterior probabilities (100%; PP=1; orange clade; Fig. 2), as well as the HBL-Hb genes that also clustered into one monophyletic group with strong statistical support (100%; PP=1; green clade; Fig. 2). When HBL-Hbs sequences from other metazoans were added to the alignment, all extracellular globins from both annelids and other metazoans clustered together in one monophyletic group with high support values (100%; Supplementary file 6).

Mbs clustered in two distinct well-supported clades (100%; PP>0.97; purple clades; Fig. 2), as well as Cygbs which also grouped into two separate highly supported clades (100%; PP>0.99; pink clades; Fig. 2). Concerning nHbs, a majority of sequences (26 sequences from a total of 39) clustered into one large and highly supported clade (100%; PP=1; blue clade; Fig. 2) and four other small clades with very few sequences in each one (blue clades; Fig. 2). The majority of Hb sequences also clustered into one large clade (67%; PP>0.58; yellow clade; Fig. 2), however several smaller clades with few sequences were also formed (yellow clades; Fig. 2). Within the Hb clades some low nodal support values were found. However, such results are common in phylogenetic analyzes within the same protein family (DeSalle, 2015). The tertiary structures the different globin genes inferred using the Phyre2 web portal
resulted in proteins with high similarity among their tertiary structure and putative respiratory function (Supplementary file 7). Model prediction confidence and coverage ranged from 97 – 100%.

In the phylogenetic reconstruction recovered with the annelid globins + metazoan globins dataset Adgbs from both vertebrates and invertebrates clustered in one clade (75%; red clade; Fig. 3), and HBL-Hbs also clustered into one monophyletic group (75%; green clade; Fig. 3). Mb clustered in two separate clades with strong bootstrap support values, one with vertebrate Mbs (85%; purple clade; Fig. 3) and another with invertebrate Mbs (100%; purple clade; Fig. 3). Cygbs formed three different groups with high support values, one with vertebrate Cygbs (85%; pink clade; Fig. 3), and two others with invertebrate Cygbs (86 and 87%; pink clades; Fig. 3). nHbs and vertebrate neuroglobins (Ngb) clustered into two distinct clades (71 and 75%; blue clades; Fig. 3), as well as vertebrate Hbs A and B, which also clustered in two monophyletic groups (95%; gray clades; Fig. 3). Invertebrate Hbs clustered into five different clades (yellow clades; Fig. 3). Some low nodal support values were also found in this gene tree.

DISCUSSION

We recovered two more types of non-circulating globins within annelids: androglobins (Adgbs) and cytoglobins (Cygbs), in addition to the already known nerve Hbs (nHbs) and myoglobins (Mbs), confirming that Annelida has the greatest diversity of oxygen-binding proteins (Mangum, 1998; Weber and Vinogradov, 2001; Costa-Paiva et al., 2017, 2018). Three annelid families, Opheliidae, Terebellidae, and Alvinellidae, were previously known to simultaneously express the three globin types – extracellular circulating, intracellular circulating and intracellular noncirculating (Weber, 1978; Hourdez et al., 2000). Our data reveal three additional families that express these three globin types: Aeolosomatidae, Arenicolidae, and Nephtyidae. In conjunction with results of Bailly et al. (2007), our results demonstrate that co-occurrence of different globin types is much more common than previously documented and that it probably already existed in the annelid ancestor. Our findings corroborate previous studies that suggest that vertebrate Cygbs and Mbs lineages are distinct from invertebrate Cygbs and Mbs lineages (Suzuki and Imai, 1998; Hoffmann et al., 2011, 2012; Blank and Burmester, 2012; Storz et al., 2013; Pillai et al., 2020). Furthermore, our phylogenetic analyses show that Cygbs and Mbs from vertebrates are not true orthologs of invertebrate Cygbs and Mbs (Fig. 3). These proteins received the same name because of their presumed functional role and not their evolutionary relationships.
The phylogenetic hypothesis for invertebrate globin sequences constructed by Goodman et al. (1988) divided annelid globins into two monophyletic groups: intracellular and extracellular. Similarly, the phylogenetic analysis carried out by Bailly et al. (2007) on the annelid globin genes demonstrated a well-supported division between intracellular and extracellular globins. Interestingly, we did not find one clade of extracellular globins and another of intracellular globins (Fig. 2). In our phylogenetic reconstruction, extracellular globins clustered into one group within a major clade containing Hbs also (Fig. 2). Based on our results, the extracellular globins appear to be phylogenetically closer to the intracellular globins. When additional extracellular globin sequences from other metazoans were added to the analysis, they all clustered together, forming a monophyletic group (Supplementary file 6). These results suggest that the extracellular globins from annelids are more closely related to the extracellular counterparts in other invertebrate phyla (Belato et al., 2019) than to the annelid intracellular globins. Studies suggest that extracellular globins arose from an ancient duplication event of an intracellular globin gene (Gotoh et al., 1987; Yuasa et al., 1996; Bailly et al., 2007; Belato et al., 2019). Considering that extracellular globins were found in both deuterostome and protostome lineages, such as Echinodermata, Mollusca, Platyhelminthes, and Brachiopoda (Belato et al., 2019), this duplication event most likely occurred at least before the protostome-deuterostome split.

Hbs are very ancient proteins and have already undergone several gene duplication events (Goodman et al., 1988; Hardison, 1998; Vinogradov et al., 2005, 2007; Storz, 2018; Pillai et al., 2020). Supporting these observations, we find that Hbs are divided into several subgroups, that may represent paralogs. Annelid Hbs clustered into one large clade that represents the annelid intracellular monomeric RBC Hbs (Goodman et al., 1975, 1988; Weber and Vinogradov, 2001; Bailly et al., 2007), and some other smaller clades with few sequences (Fig. 2, yellow clades). Moreover, as expected, Hbs from G. dibranchiata and G. americana clustered into one separate clade compared to other Hbs, which seems to represent the well-known distinct circulating glycerid RBC Hbs (Weber et al., 1977, Weber and Vinogradov, 2001).

Herein we present the first record of Adgbs in annelids, with all newly discovered sequences clustered into one well supported group (Fig. 2, orange clade), which is consistent with their conspicuous inversion in the globin domain, with helices C-H preceding helices A-B (Hoogewijs et al., 2012; Bracke et al., 2018). In the gene tree of annelid globins + metazoan globins, the Adgbs of vertebrates and invertebrates clustered together, corroborating the hypothesis that Adgb genes predate the origin of
metazoans (Hoogewijs et al., 2012; Bracke et al., 2018). We also present the first record of invertebrate Cygbs within the Annelida. Cygbs grouped into two distinct clades (Fig. 2, pink clades), where one is a sister group to nHbs and the other is sister group to a clade that contains Mbs and Hbs (Fig. 2). These results indicate that Cygbs appear to have a high molecular affinity to other non-circulating intracellular globins, such as invertebrate Mbs and nHbs, similar to vertebrate Cygbs, which show phylogenetic affinities to vertebrate Mbs (Burmester et al., 2002). Annelid Mbs clustered into two well-supported clades (Fig. 2, purple clades), and these two distinct groups presumably represent the two major components MbI and MbII that have been reported in the annelid Arenicola marina (Weber and Pauptit, 1972; Kleinschmidt and Weber, 1998). Our results are in agreement with those from Suzuki and Imai (1998), which separated Mbs from invertebrates and vertebrates (Fig. 3; purple clades).

Some nerve Hbs clustered into one big clade (Fig. 2, blue clade) with high support values, and the other ones are mixed with other intracellular Hbs sequences (Fig. 2, yellow and blue clades), supporting the hypothesis of divergent evolutionary origins of invertebrate nHbs (Wittenberg, 1992; Weber and Vinogradov, 2001). Dewilde et al. (1996) and Burmester et al. (2000) have reported that nHbs from the worm Aphrodita aculeata reveal higher sequence similarity to the intracellular Hbs from the bloodworm Glycera dibranchiata than to other invertebrate nHbs. Our genealogy corroborates these results in that the nHbs from Aphrodita japonica are more closely related to the Hbs from G. dibranchiata than to the other nHbs grouped in the bigger clade (Fig. 2). Vertebrate neuroglobins and nHbs clustered in separate clades (Fig. 3), confirming that although both globins are localized in nerve tissues, they have different evolutionary origins (Weber and Vinogradov, 2001; Blank and Burmester, 2012; Pillai et al., 2020). The inferred tertiary structures of the globin genes suggested that they could have a putative respiratory function (Supplementary file 7).

In conclusion, our findings confirm a pattern evident from several recent studies, there is much greater phylogenetic distribution of oxygen-binding proteins than previously established, especially in annelids (Bailly et al., 2008; Martín-Durán et al., 2013; Costa-Paiva et al., 2017, 2018; Belato et al., 2019). We found two new intracellular non-circulating globin types within annelids: Adgbs and Cygbs, in addition to the two other documented types. We confirm that Mbs and Cygbs from vertebrates and those from invertebrates have different evolutionary origins. Our analyses demonstrate an intimate relationship between annelid extracellular globins and those from other metazoans, most likely because they were already present in the common ancestor of protostomes and deuterostomes — and reaffirm the crucial
importance of further comprehensive studies on the molecular evolution of the globin super-family across the metazoan evolutionary tree.

DATA DEPOSITION
This project has been deposited at GenBank under accession numbers MT311987 to MT312212.

COMPETING INTERESTS
The authors declare that they have no competing interests.

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FIGURE LEGENDS

Figure 1 – Multiple amino acid sequence alignment of annelid Cygb (cytoglobin), Hb (hemoglobin), HBL (hexagonal bilayer hemoglobin), nHb (nerve hemoglobin), Mb (myoglobin), and manually rearranged Adgb (androglobin). Invariant amino acid residues at positions CD1, E7 and F8, which are diagnostic characters of the globin domain, are indicated in bold.

Figure 2 – Maximum likelihood gene genealogy of annelid globin genes rooted by midpoint. Bootstrap support values obtained from the maximum likelihood inference are shown in black, and the posterior
probabilities values obtained from the Bayesian inference are shown in red. To improve clarity, only support values above 80 or 0.8 are shown. Posterior probabilities values Yellow clades represent hemoglobin groups. Blue clades represent nerve hemoglobins. Purple clades represent myoglobins. Green clade represents hexagonal bilayer hemoglobins. Pink clades represent cytoglobins. Orange clade represents androglobins.

**Figure 3** – Maximum likelihood gene genealogy of 43 annelid globin genes and 54 metazoan globin genes rooted by midpoint. Bootstrap support values obtained from the maximum likelihood inference are shown above the branches. Dark and light blue clades are nerve hemoglobin and vertebrate neuroglobin, respectively. Green clade is hexagonal bilayer hemoglobin. Yellow clades are invertebrate hemoglobins. Dark and light pink clades are invertebrate and vertebrate cytoglobin, respectively. Dark and light gray clades are vertebrate hemoglobin A and B. Red clade is androglobin. Dark and light purple clades are vertebrate and invertebrate myoglobin, respectively. Vertebrate and invertebrate Cygbs, Hbs, and Mbs do not represent genuine orthologs.

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Table 1 – List of all taxa analyzed in which globin genes were found and the number of expressed genes in each species. Hb is hemoglobin, Mb is myoglobin, nHb is nerve hemoglobin, Cygb is cytoglobin, Adgb is androglobin, and HBL-Hb is hexagonal bilayer hemoglobin. GenBank accession numbers are also provided here and detailed in Supplementary file 4.

| Taxon                      | Hb genes | Mb genes | nHb genes | Cygb genes | Adgb genes | HBL-Hb genes | Accession number |
|----------------------------|----------|----------|-----------|------------|------------|--------------|-----------------|
| Acrodiridae                |          |          |           |            |            |              |                 |
| *Macrochaeta* sp.          |          |          |           |            |            |              |                 |
| Aeolosomatidae             |          |          |           |            |            |              |                 |
| *Aeolosoma* sp.            |          |          |           |            |            |              |                 |
| Alciopidae                 |          |          |           |            |            |              |                 |
| *Alciopa* sp.              |          |          |           |            |            |              |                 |
| Alvinellidae               |          |          |           |            |            |              |                 |
| *Paralvinella palmiformis* |          |          |           |            |            |              |                 |
| Desbruyères & Laubier, 1986|          |          |           |            |            |              |                 |
| *Paramphinome jeffreysii*  |          |          |           |            |            |              |                 |
| (McIntosh, 1868)           |          |          |           |            |            |              |                 |
| Ampharetidae               |          |          |           |            |            |              |                 |
| *Amphisamytha galapagensis*|          |          |           |            |            |              |                 |
| Zottoli, 1983              |          |          |           |            |            |              |                 |
| *Auchenoplatx crinita*     |          |          |           |            |            |              |                 |
| Ehlers, 1887               |          |          |           |            |            |              |                 |
| *Melinna maculata*         |          |          |           |            |            |              |                 |
| Webster, 1879              |          |          |           |            |            |              |                 |
| Amphinomidae               |          |          |           |            |            |              |                 |
| *Chloeia pinnata* Moore, 1911|      |          |           |            |            |              | MT312090         |
| *Hermodice carunculata*    |          |          |           |            |            |              | MT312103         |
| (Pallas, 1766)             |          |          |           |            |            |              |                 |
| *Pherecardia striata*      |          |          |           |            |            |              | MT312152         |
| (Kinberg, 1857)            |          |          |           |            |            |              | MT312032         |
| Aphroditidae               |          |          |           |            |            |              |                 |

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Aphrodita japonica Marenzeller, 1879

Arenicolidae
Arenicola loveni Kinberg, 1866

Abarenicola pacifica Healy & Wells, 1959

Aspidosiphonidae
Aspidosiphon laevis Quatrefages, 1865

Branchiobdellidae
Branchiobdella parasita (Braun, 1805)

Chaeotrichidae
Chaeotrichus variopedatus (Renier, 1804)

Mesochaetopterus taylori Potts, 1914

Chrysoptilidae
Arichlidon gathofi Watson Russell, 2000

Cirratulidae
Aphelochaeta sp.

Chaetozone sp.

Tharyx kirkegaardii Blake, 1991

Dinophilidae
Dinophilus gyrociatus O. Schmidt, 1857

Dorvilleidae
Ophryotrocha globopalpata Blake & Hilbig, 1990

Eunicidae
Eunice norvegica (Linnaeus, 1767)

Eunice pennata (Müller, 1776)

Marphysa sanguinea (Montagu, 1813)

Palola sp.

Flabelligeridae
Ilyphagus octobranchus Hartman, 1965

Poeobius meseres Heath, 1930

Glossoscolecidae
Andiorrhinus sp.

Pontoscolex corethrurus (Muller, 1857)

Urobenus sp.

Glyceridae
Glycera americana Leidy, 1855

Glycera dibranchiata Ehlers, 1868

Hemipodia simplex (Grube, 1857)

Goniadidae
Goniada brunnea Treadwell, 1906

Haplotaxidae
Delaya leruthi (Hrabê, 1958)

Haplotaxidae gen. sp.

Hesionidae
| Family          | Genus                     | Species                          | Year     | MT Numbers |
|-----------------|---------------------------|----------------------------------|----------|------------|
| Hesionides      |                           | sp.                              | 11967    | MT312129   |
| Microphthalmus  | listenis Westheide        | 1                                | MT312153 |
| Microphthalmus  | similis Bobretzky, 1870   | 1                                | MT312070 |
| Histriobdellidae|                           |                                  | MT312033 |
| Microphthalmus  | homari Beneden, 1858      | 2                                | MT312063 |
| Histriobdella   |                           |                                  | MT312064 |
| Hrabeiellidae   |                           |                                  | MT312065 |
| Komarekionidae  |                           | 1                                | MT312028 |
| Lessoniaceae    |                           |                                  | MT312029 |
| Eisenia         | sp.                       | 1                                | MT312030 |
| Lumbricidae     |                           |                                  | MT312031 |
| Dendrobaena     | hortensis (Michaelsen, 1890) | 1                                | MT312032 |
| Lumbrineridae   |                           |                                  | MT312033 |
| Lumbrineris     | crassicephala Hartman, 1965 | 1                                | MT312034 |
| Umoe nigripes    | Verrill, 1873             | 2                                | MT312035 |
| Maldanidae      |                           |                                  | MT312036 |
| Axiothella      | rubrocincta (Johnson, 1901) | 1                                | MT312037 |
| Clymenella      | torquata (Leidy, 1855)    | 1                                | MT312038 |
| Nicomache       | venticola Blake & Hilbig, 1990 | 1                                | MT312039 |
| Praxillella     | pacifica Berkley, 1929    | 1                                | MT312040 |
| Sabaco elongatus| Verrill, 1873             | 2                                | MT312041 |
| Megascoleidae   |                           |                                  | MT312042 |
| Amynthas        | sp.                       | 1                                | MT312043 |
| Pontodrilus     | litoralis (Grube, 1855)   | 1                                | MT312044 |
| Microchaetidae  |                           |                                  | MT312045 |
| Gattyana        | cirrhosa (Pallas, 1766)   | 1                                | MT312046 |
| Kynotus         | pittarelli Cognetti, 1906 | 1                                | MT312047 |
| Moniligastridae |                           |                                  | MT312048 |
| Drawida         | sp.                       | 1                                | MT312049 |
| Naididae        |                           |                                  | MT312050 |
| Aulodrilus      | japonicus Yamaguchi, 1953 | 1                                | MT312051 |
| Bothrioneurum   | vejdoskynyanu Stolc, 1886 | 1                                | MT312052 |
| Heterodrilus    | sp. 1                     | 1                                | MT312053 |
| Nephtyidae      |                           |                                  | MT312054 |
| Aglaophamus     | verrilli (McIntosh, 1885) | 1                                | MT312055 |

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| Family               | Genus                        | Species                                  | Author       | MT312099-101 | MT312100-101 |
|----------------------|------------------------------|------------------------------------------|--------------|--------------|--------------|
| Nereididae           | Nephtys incisa              | Malmgren, 1865                           | MT312156     | MT312071     |              |
| Alitta succinea      | (Leuckart, 1847)            |                                          | MT312089     |              |              |
| Octochaetidae        | Dichogaster green tree worm  |                                          | MT312022     | MT312023     |              |
|                      | Dichogaster guadeloupensis  | James, 1996                              | MT312094     | MT312004     |              |
| Oenonidae            | Arabella sp.                |                                          |              |              |              |
|                      | Drilonereis sp.             |                                          |              |              |              |
| Onuphidae            | Diopatra cuprea             | (Bosc, 1802)                             | MT312138-39  | MT312067-68  |              |
|                      | Armandia sp.                |                                          | MT312155     | MT312179     |              |
| Opheliidae           | Ophelina acuminata          | Örsted, 1843                             | MT312118-19  | MT312035     |              |
|                      |                            |                                          |              |              |              |
| Octochaetidae        | Naineris laevigata          | (Grube, 1855)                            | MT312194-96  | MT312081     |              |
|                      | Proscoloplos cygnochaetus    | Day, 1954                                | MT312170     |              |              |
| Oeniidae             | Galathowenia oculata         | (Zachs, 1923)                            |              |              |              |
|                      | Owenia fusiformis            | Delle Chiaje, 1844                      |              |              |              |
| Parergodrilidae      | Stygocapitella subterranea   | Knöllner, 1934                           | MT312194-96  | MT312081     |              |
|                      |                            |                                          |              |              |              |
| Parvidrilidae        | Parvidrilus meyssonnier      | DesChâtelliers & Martin, 2012            | MT312101     |              |              |
| Pectinariidae        | Pectinaria gouldii           | (Verrill, 1874)                          | MT312055     | MT312027     |              |
| Phyllodocidae        | Eulalia myriacyclum          | (Schmarda, 1861)                         | MT312157     |              |              |
|                      | Nereiphylla sp.             |                                          |              |              |              |
| Pilargidae           | Synelmis sp.                |                                          | MT312197     |              |              |
| Polygordiidae        | Polygordius sp.             |                                          | MT312074     |              |              |
| Polynoidae           | Halosyndra brevisetosa      | Kinberg, 1855                            | MT312058     |              |              |
|                      | Hermenia verruculosa        | Grube, 1856                              | MT312060-61  |              |              |
|                      | Lepidonotus semitectus       | Stimpson, 1856                           | MT312030     |              |              |
| Protodriloididae     | Protodriloides chaetifer     | Remane, 1926                             | MT312180-83  | MT312077     |              |
| Sabellariidae        | Idanthyrus sp.              |                                          | MT312131-32  |              |              |
| Sabellidae           | Bispira pacifica            | (Berkeley & Berkeley, 1954)             | MT311989     | MT312154     |              |
|                      | Myxicola infundibulum       | (Montagu, 1808)                          | MT312186     |              |              |
| Scalibregmatidae     | Scalibregma inflatum        | Rathke, 1843                             |              |              |              |
| Serpulidae           |                            |                                          |              |              |              |

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Crucigera zygophora (Johnson, 1901) 2
Galeolaria caespitosa Lamarck, 1818 1
Serpula vermicularis Linnaeus, 1767 1
Spirobranchus kraussii (Baird, 1865) 2

Siboglinidae

Lamellibrachia luymesi van der Land & Nørrevang, 1975 1
Osedax sp. 2
Sclerolinum brattstromi Webb, 1964 1
Siboglinum ekmani Jägersten, 1956 3

Sigalionidae

Sigalion sp. 1

Sparganophilidae

Sparganophilus sp. 1

Spionidae

Boccardia proboscidea Hartman, 1940 2
Laonice sp. 2
Prionospio dubia Day, 1961 1

Sternaspidae

Sternaspis scutata (Ranzani, 1817) 1
Sternaspis sp. 1

Syllidae

Odontosyllis gibba Claparède, 1863 1
Syllis cf. hyalina Grube, 1863 1

Terebellidae

Eupolyminia nebulosa (Montagu, 1819) 3
Lanicides sp. 4
Lysilla sp. 1

Pista macrolobata Hessle, 1917 4
Streblosoma hartmanae Kritzler, 1971 2
Terebellides stroemii Sars, 1835 8
Thelepus crispus Johnson, 1901 2

Themistidae

Themiste pyroides (Chamberlin, 1919) 1

TravisIIDae

Travisia brevis Moore, 1923 3

Tritogeniidae

Tritogena sulcata Kinberg, 1867 1

Trochochaetidae

Trochochaetidae gen. sp. 1
Figure 1 – Multiple amino acid sequence alignment of annelid Cygb (cytoglobin), Hb (hemoglobin), HBL (hexagonal bilayer hemoglobin), nHb (nerve hemoglobin), Mb (myoglobin), and manually rearranged Adgb (androglobin). Invariant amino acid residues at positions CD1, E7 and F8, which are diagnostic characters of the globin domain, are indicated in bold.
Figure 2 – Maximum likelihood gene genealogy of annelid globin genes rooted by midpoint. Bootstrap support values obtained from the maximum likelihood inference are shown in black, and the posterior probabilities values obtained from the Bayesian inference are shown in red. To improve clarity, only support values above 80 or 0.8 are shown. Posterior probabilities values Yellow clades represent hemoglobin groups. Blue clades represent nerve hemoglobins. Purple clades represent myoglobins. Green clade represents hexagonal bilayer hemoglobins. Pink clades represent cytoglobins. Orange clade represents androglobins.
Figure 3 – Maximum likelihood gene genealogy of 43 annelid globin genes and 54 metazoan globin genes rooted by midpoint. Bootstrap support values obtained from the maximum likelihood inference are shown above the branches. Dark and light blue clades are nerve hemoglobin and vertebrate neuroglobin, respectively. Green clade is hexagonal bilayer hemoglobin. Yellow clades are invertebrate hemoglobins. Dark and light pink clades are invertebrate and vertebrate cytoglobin, respectively. Dark and light gray clades are vertebrate hemoglobin A and B. Red clade is androglobin. Dark and light purple clades are vertebrate and invertebrate myoglobin, respectively. Vertebrate and invertebrate Cygbs, Hbs, and Mbs do not represent genuine orthologs.