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Protein and Gene Structure of a Chlorocruorin Chain of *Eudistylia vancouverii*

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The polychaete annelid, *Eudistylia vancouverii*, contains as oxygen carrier a hexagonal bilayer (HBL) chlorocruorin. One of the globin chains, chain a1, has 142 amino acids (Mr 16,054.99) and its sequence deviates strongly from other nonvertebrate globin sequences. Unprecedented, it displays a Phe at the distal position E7 as well as at position B10, creating a very hydrophobic heme pocket probably responsible for the low oxygen affinity of the native molecule. Phylogenetic analysis of annelid globin chains clearly proves that globin chain a1 belongs to type I of globin chains having a pattern of 3 cysteine residues essential for the aggregation into a HBL structure. The gene coding for globin chain a1 is interrupted by 2 introns at the conserved positions B12.2 and G7.0. Based on protein and gene structure it can therefore be concluded that the globin chains of chlorocruorins are not fundamentally different from other annelid globin chains.

Key Words: **chlorocruorin; globin chain; *Eudistylia vancouverii;* HLB structure.**

Chlorocruorins (Chls) are giant, extracellular oxygen-binding heme proteins found in four marine polychaete families, which contain an altered heme with a formyl substituting for the 3-vinyl group and consequently appear as greenish-red (1). The Chls have the hexagonal bilayer (HBL) appearance and size in electron micrographs characteristic of annelid and vestimentiferan extracellular Hbs, a similar sedimentation coefficient of approximately 60S and an abnormally low iron content of 0.23 wt.% (2–6). Although the oxygen binding affinities of the Chls are generally lower than those of the annelid Hbs (P_{50} ~150 torr at neutral pH), the Chls exhibit equally high cooperativities (Hill coefficient n50 > 3) (7–10).

The HBL Hbs from various annelid groups and the vestimentiferans and Chls differ among themselves in the nature of their disulfide-bonded globin subunits. The majority of oligochaete and polychaete Hbs have monomers and disulfide-bonded trimers, the leeches and the vestimentiferans have monomers and disulfide-bonded dimers and the Chls have disulfide-bonded dimers and tetramers (5, 11, 12).

As a result of several extensive studies over the last 20 years, the HBL Hbs and Chls are known to have masses of ~3600 kDa and to consist of two types of chains, heme-containing 16–17 kDa globin chains and nonglobin, linker chains of 25–32 kDa in an approximate 2:1 molar ratio (1). All the HBL Hbs including the Chls have almost identical three-dimensional structures determined by cryoelectron microscopy at about 3 nm resolution by J. Lamy and his collaborators (13–17). Recent detailed studies of the dissociation of *Eudistylia vancouverii* Chl and of its constituent chains and subunits by electrospray ionization mass spectrometry (18, 19) have shown it to consist of two types of globin subassemblies, a dodecamer formed by the noncovalent association of three disulfide-bonded trimers and a tetramer formed by the noncovalent association of disulfide bonded dimers. So far it is the most complicated HBL structure known (Tables 1–3).

This communication presents the complete amino acid sequence of one of the six globin chains of *Eudistylia* Chl and the structure of its corresponding gene.

MATERIALS AND METHODS

Determination of the primary structure of a globin chain at the protein level. Live *Eudistylia vancouverii* were collected at the Oregon Institute of Marine Biology (Charleston, OR) and the Chl was prepared as described previously in 0.1M Tris.HCl buffer pH 7.0, 1

Sequence reported in this paper was submitted to GenBank with Accession No. AF257469.

Abbreviations used: Chl, chlorocruorin; Glb-a1, globin chain a1; HBL, hexagonal bilayer; Mb, myoglobin; Hb, hemoglobin; MMLV-RT, Molony murine leukemia virus reverse transcriptase.

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TABLE 1

Characteristics of *E. vancouverii* Chlorocruorin: The Native Molecule and Its Subunits (13, 18, 36–39)

Mr	3.48 ± 225 kDa (Anal. Ultracentrif.)
Haem	Chlorocruoro haem (formyl in position 3)
Dimensions	27.5 nm × 18.5 nm (STEM)
Quaternairy structure	Hexagonal bilayer appearance (HBL) (E.M. + Cryo-E.M.)

mM EDTA, using a cocktail of protease inhibitors (#1697–498, Biochemical Div., Boehringer Mannheim Corp., Indianapolis, IN) (18). Globin and linker chains were separated by RP-HPLC on a Synchropak RP1000 C18 column in a 0.1% TFA/acetonitrile gradient system (19). Purity was checked by one and two dimensional electrophoresis (20).

Final purification of the globin chains was performed by semipreparative isoelectro-focusing on immobilized pH gradient gel strips (pH 4–7) under denaturing conditions. The band of interest was excised, washed with distilled water and extracted with 6M urea, 0.1% TFA and vacuum-dried. Ampholites and matrix compounds were eliminated by RP-HPLC on a Vydac C4 column in a 0.1% TFA/acetonitrile gradient system (21).

The purified globin chain was separately digested with trypsin and endoprotease AspN. Resulting peptides were separated with RP-HPLC using a Vydac C4 column developed in a 0.1% TFA/acetonitrile system. The peptides were sequenced on an ABI 471-B sequencer operated as recommended by the manufacture.

Determination of the globin cDNA and genomic DNA sequence. Two primers were designed based on the obtained protein sequence data. Primer Eud3, GARGAYTTYGARGAYGAR a 18-mer with 64 redundancies, corresponding to the sense strand predicted by the peptide fragment EDFEDE. Eud2, GCRTTCCANGCRTCYTTRTC, a 20-mer with 64 redundancies, corresponding to the antisense strand predicted by the peptide fragment DKDAWNA.

Total RNA was isolated from the body wall of *E. vancouverii* using the Total RNA isolation kit from Stratagene. First strand cDNA was synthesized with MMLV-RT (Promega) using random hexamers or oligodT primer.

A PCR reaction was then performed for 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min using the degenerate primers. The amplified product was purified with a PCR purification kit (Boehringer) and sequenced (cycle sequencing) on an automated ABI 377 sequencer.

Specific primers EudF6, GTGCAGAGTTCAATTTGGCA, EudR4, ATCCAACCCACTAGCCACAC and EudR5, GCACGGAATTCTGGGGAGTA, were designed based on the obtained DNA sequence. EudF6 and oligodT were used in a PCR to obtain the 3′ end of the cDNA (30 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C). Amplified product was purified and sequenced as described above. A RACE was carried out to obtain the 5′ end of the cDNA. First strand cDNA was synthesized using EudR4. A poly(C) tail was added to the end of the cDNA with terminal deoxynucleotide transferase. A PCR was then carried out using an oligodG adapter and the specific nested primer EudR5 (30 cycles of 1 min at 94°C, 1 min at 57°C, and 2 min at 72°C). Positive amplification products were purified and sequenced as described above.

Based on the full cDNA sequence 3 additional primers were designed. The forward primers EudF7, TGCTAGGAGTTGGTCAGCCT and EudF10, CGTGTCCGTGGAGATA ACAT and the reverse primer EudR8, AAAATTGGTTGACTTTTCTGCAA.

Isolated gDNA was used as template in three PCR reactions (30 s at 94°C, 1 min at 53°C, and 5 min at 72°C for 35 cycles). EudF7 was used as a forward primer with EudR4 and EudR5 as reverse primers respectively. Amplification products were purified on agarose gel and sequenced. A third PCR was done using the primers EudF10 and EudR8. The amplified product was purified and sequenced as described above.

Alignment and tree construction. Thirty-two annelid globin sequences were aligned manually using globin landmarks and the nonvertebrate globin template (22) as guides. Based on this alignment a neighbor-joining tree was constructed using the TREECON software (23).

RESULTS AND DISCUSSION

Primary Structure of Globin a1

Separation of the constitutive chains of purified *E. vancouverii* Chl by RP-HPLC results in 4 globin peaks (Fig. 1; G1–4). Each peak contains multiple globin chains as shown by mass spectrometry (19), (Table 2) and one and two dimensional electrophoresis (Fig. 2). Fraction G4 was selected for further purification of a globin chain by semipreparative isoelectric focusing (21).

The primary structure of one of these globin chains was reconstructed from the sequence of informative peptides obtained by tryptic and endoprotease Asp-N digestion. All overlaps are documented (Fig. 3).

Full-length cDNA was isolated and sequenced as described (Fig. 4). It encompasses the entire coding region and confirms the amino acid sequence determined at the protein level. The open reading frame extends for 142 codons and is preceded by a signal peptide of 18 codons. A 5′ untranslated region of 109 bases and a 3′ untranslated region of 516 bases is present as well as a normal polyadenylation signal. From the sequence, a mass of 16,054.99 was calculated. This allows the identification of this globin chain as Glb-a1 according to the nomenclature of Green *et al.* 1998 (19) (Table 2).

Glb-a1 was aligned with 32 annelid, pogonophoran and vestimentiferan globin sequences available in our database including a globin chain from the Chl of *Sabelastarte indica* (24) (alignment available from the authors; a reduced representative alignment is presented in Fig. 3). The alignment of Glb-a1 is unambiguous due to the presence of globin landmarks A12-Trp, C2-Pro, CD1-Phe, F8-His, and H8-Trp. The total penalty score against the nonvertebrate template is 7.2 indicating deviations of the standard pattern (penalty scores available upon request) (22). At the surface positions A6, A10, CD2 and F3 hydrophobic residues are observed whereas at the internal position G5 a hydrophilic residue occurs. As similar, hydrophobic substitutions occur in other annelid globin sequences it is most likely that they represent specific adaptations for the aggregation into a high Mr complex. In addition at position E11 a residue with a small side chain (Val) occurs whereas a larger site chain is expected. No specific adaptation can be localized to harbor the formyl group on the heme ring.

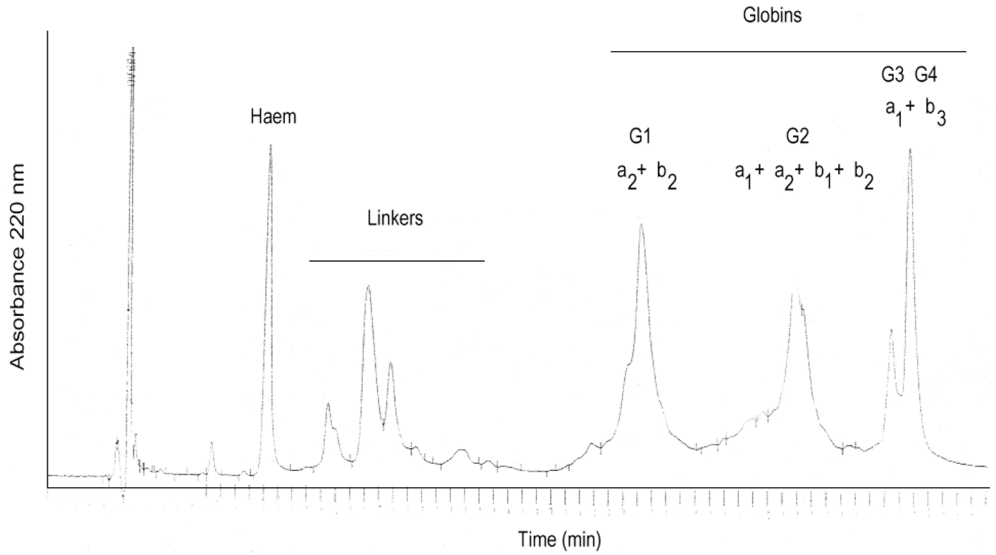

FIG. 1. Separation of *E. vancouverii* globin and linker chains by RP-HPLC. Separation was performed on a 4.6 × 250 mm C18 Synchropak RP-P column and developed with two consecutive gradients of acetonitrile in aqueous 0.1% TFA as described [19].

The most striking feature of the Glb-a1 sequence, however, is the unprecedented occurrence of Phe at the distal position. This creates together with B10-Phe a very hydrophobic distal side with no residue able to form a hydrogen bond with the bound oxygen. Stabilisation of the oxygen might therefore occur, as in *Aplysia* Mb, by the E10-Arg (25–27) (Fig. 3). *E. vancouverii* Chl has a low oxygen affinity which might be counted by similar substitutions in one or more globin chains (9) (Table 3). Indeed a recombinant Phe(beta)E7 HbA shows a low-affinity, noncooperative oxygen binding to the alpha subunits.

It has to be noted that the only other globin chain of a chlorocruorin known today (*S. indica),* has a penalty score of 1.5 and thus fits much better with the template. There is no direct explanation for this difference.

TABLE 2

Globin Chain Composition (19)

HPLC	Chain	Mass	Ratio	Cys
	Monomers	(±1Da)		
	A1	16,051.5	8	3
	A2	16,172.4	4	3
	B1	16,853.5	1	5
	B2	17,088.9	4	4
	B3	17,161.2	2	4
	B4	17,103.6	1	nd
	Dimers	(±3Da)		
G3 + G4	D1 = a1b3	33,207.1	5	
G1	D2 = a2b2	33,374.1	4	
	D3 = a1b4	33,149.4	1	
	Tetramers	(±4Da)		
G2	T = a1a2b1b2	66,154.8		

The homology between the *E. vancouverii* and *S. indica* is 31.5% whereas the rest of the sequences display a homology between ~18 (*Glycera* P1) to 44% (*Lumbricus* D2).

A neigbor-joining tree, constructed using the annelid, pogonophoran, and vestimentiferan globin se-

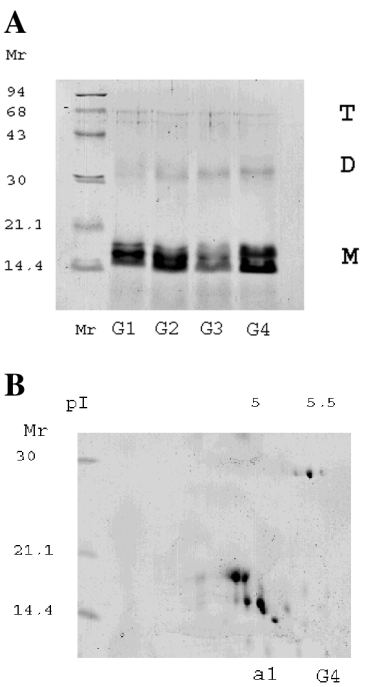

FIG. 2. Analysis of globin fractions. (A) One dimensional SDS–PAGE of the globin fractions obtained in Fig. 1. (B) Two dimensional electrophoresis of globin fraction G4. Mr, Mr markers; G1–G4, globin fraction 1–4; a1, globin chain a1; T, tetramer; D, dimer; M, monomer.

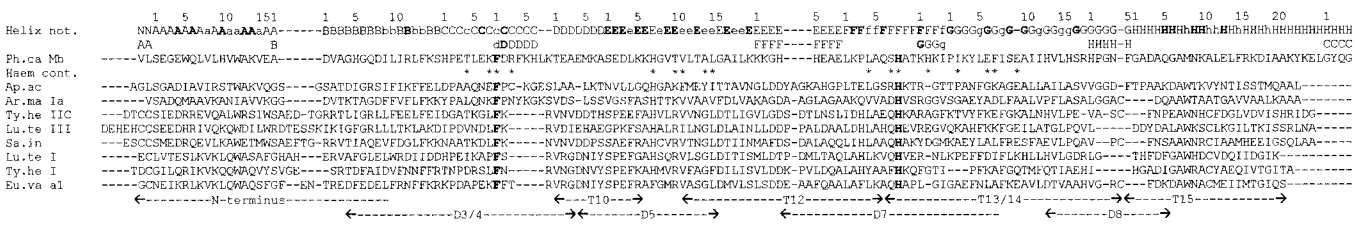

FIG. 3. Alignment of *E. vancouverii* Glb-a1 with selected sequences. The reconstruction of the Glb-a1 sequence from N-terminal sequence data and the sequence of informative peptides generated by tryptic (T) and endoprotease Asp-N (D) digestion is indicated. Ph.ca, *Physeter catodon;* Ap.ac, *Aphrodite aculeata;* Ar.ma, *Arenicola marina;* Ty.he, *Tylorrhynchus heterochaetus;* Lu.te, *Lumbricus terrestris;* Sa.in, *Sabelastarte indica;* Eu.va, *Eudistylia vancouverii.* The helix notification of sperm whale Mb is given in the heading. Surface and internal sites are given in bold and small caps respectively [34]. Heme contacts are marked * [35].

quence alignment, is shown in Fig. 5. It is evident that this tree is a gene tree rather than a phylogenetic tree (20). Three major clusters can be identified based on two major features: (i) A first feature is the intra- or extracellular nature of the Hbs which is based on the tendency of the globin chains to aggregate or not; (ii) Within the extracellular Hbs two sub-clusters can be identified based on the conservation of their cysteine patterns. Type 1 has 3 cysteines at positions NA2, GH4′ and H11, whereas type 2 displays an additional cysteine at position NA1 (Fig.3).

E. vancouverii Glb-a1 clearly belongs to type 1 containing 3 cysteine residues. Two cysteines are involved in an intra-chain disulphide bridge (19). NA2 is close to

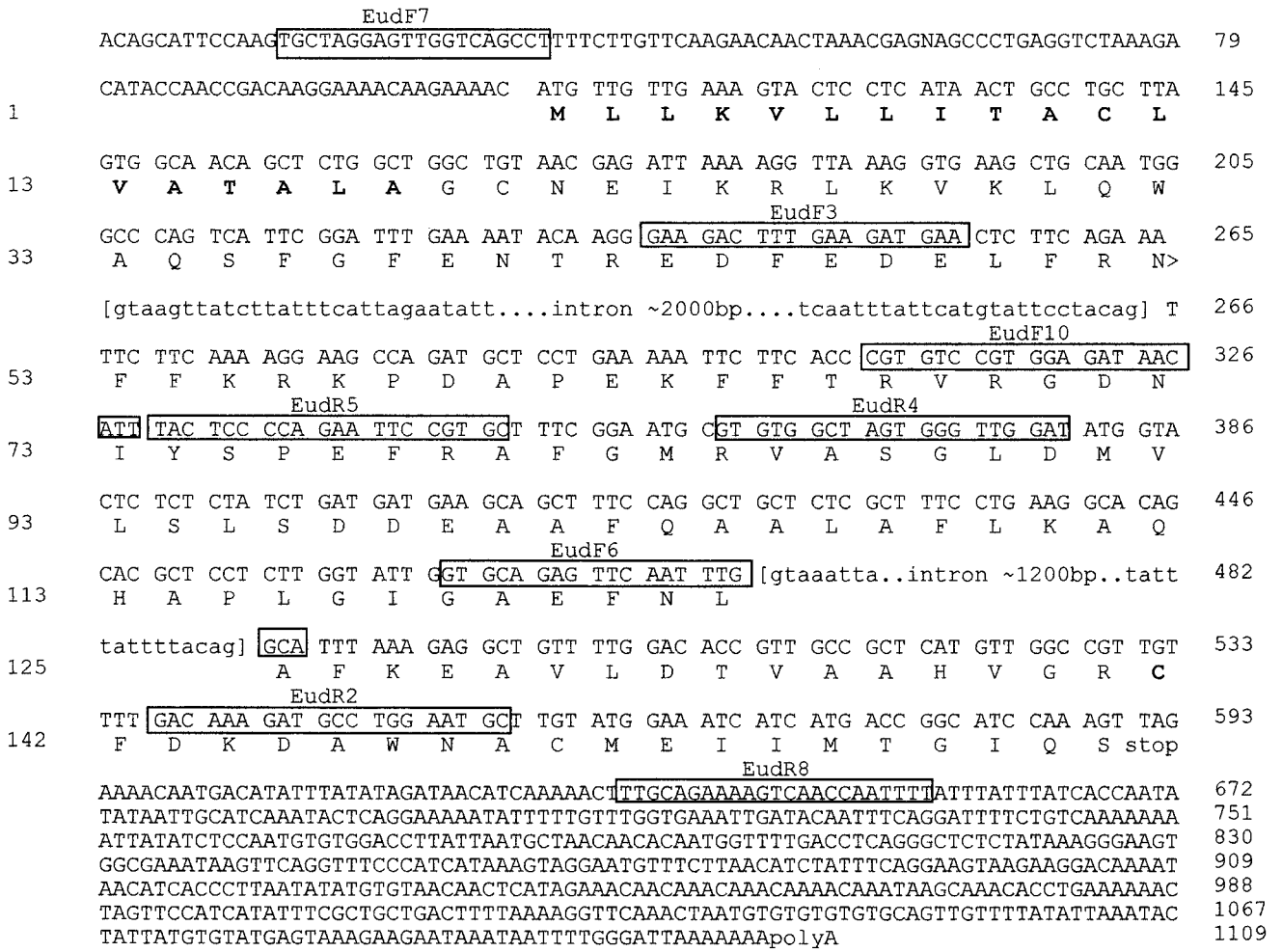

FIG. 4. cDNA and gDNA sequence of *E. vancouverii* Glb-a1. cDNA and gDNA of *E. vancouverii* Glb-a1 was determined as described under Materials and Methods. Primers are boxed; EudF are forward primers, EudR are reverse primers.

TABLE 3

Physiological Characteristics (27)

Native molecule	
P_{50}	145 mm Hg pH 7.1 25°C
N_{max}	6.9
Ø	−0.44
Dodecamer	
P_{50}	58 mm Hg
N_{max}	1.7
Ø	−0.22

H11 therefore it is most likely that they are responsible for the formation of the intra-chain disulphide bridge, linking the NA terminus to the H-helix and leaving GH4′ and NA1 for inter-chain bonds. A similar situation occurs in the other annelid, pogonophoran and vestimentiferan extracellular globin sequences (28–31). Besides in annelids, intermolecular coupling of globin chains by cysteines in the NA region is also observed in *Daphnia* where the first amino acid is a unique cysteine linking both two-domain globin chains together in a pseudo-tetramer structure (32). Next to these 3 conserved cysteines other cysteine residues are observed at the positions CD3 (1×), E8 (4×), E18 (3×), and G11 (2×). These are, most likely, free cysteines which might be involved in detoxification of toxic compounds as NO and sulphide (33). Whether this is a genuine function of the Hb or just a fortuitous effect of a reactive thiol group has to be demonstrated.

It can thus be concluded that the globin sequence of *E. vancouverii,* with exception of the unprecedented distal Phe is similar to the other annelid, pogonophoran, and vestimentiferan globin sequences and that

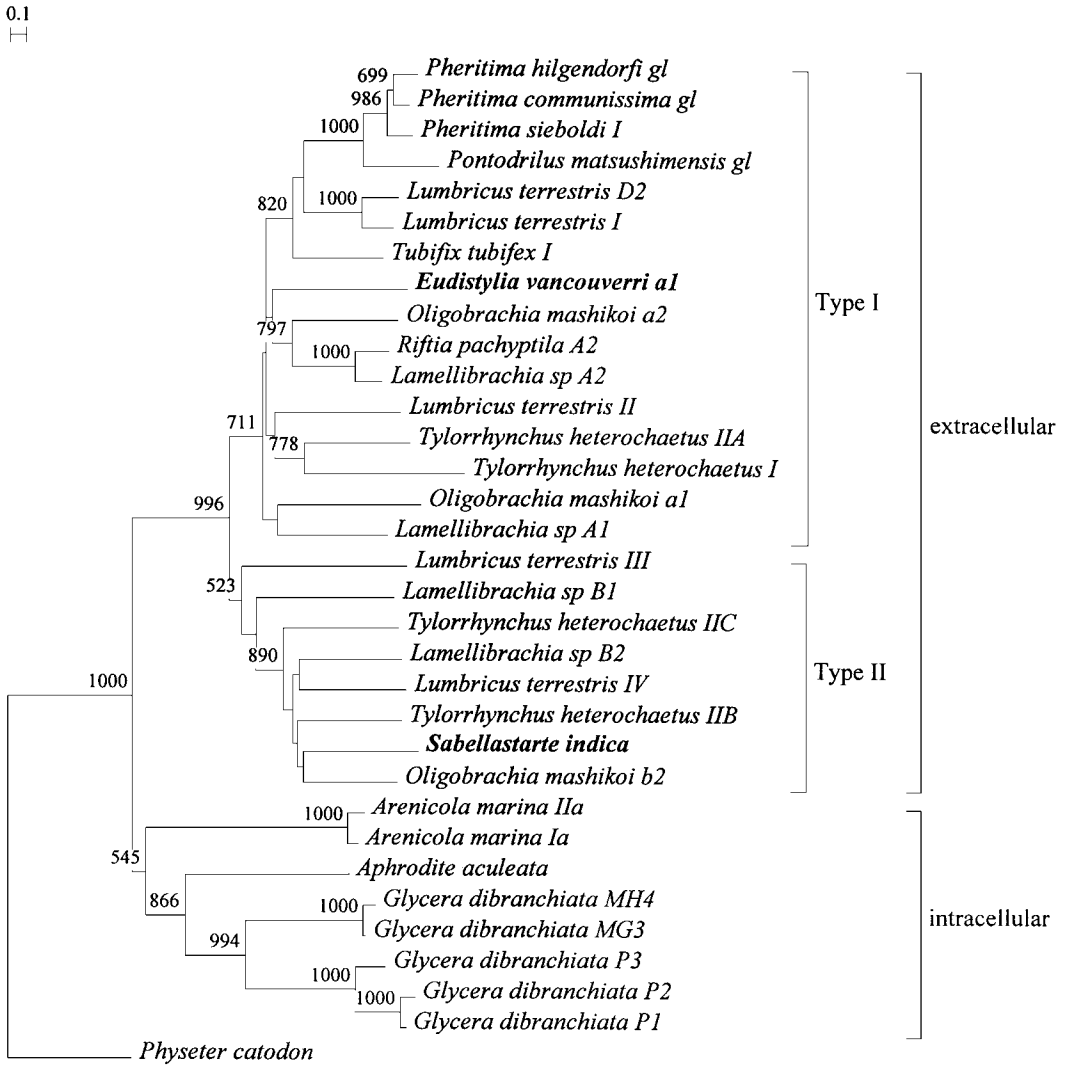

FIG. 5. Phylogenetic tree of annelid globin sequences.

there are no specific adaptations to harbor the formyl group of the heme ring, typical of a chlorocruorin.

Gene Structure of Globin Chain a1

Using cDNA derived primers the complete gene structure was obtained from two overlapping fragments. The globin gene structure contains two introns and three exons. The introns are inserted at the conserved positions B12.2 and G7.0 and have a length of, respectively, ~2000 and ~1200 bases (Figs. 3 and 4). As such the typical gene structure that is seen in all vertebrates and in most annelids (with exception of *Aphrodite aculeata* that has only one intron inserted at position G7.0) is also conserved in a chlorocruorin gene.

However it is possible that more than one gene encodes the same globin chain. Indeed two fragments were obtained in a PCR using the primers EudF7 (situated in the 5′ untranslated region) and EudR4 or EudR5 (situated in the E-helix) with a length of respectively 2300 and 2900 base pairs. Both contain the B12.2 intron and code for the same amino acid sequence. The only difference seems to be a few base substitutions in and the length of the intron sequence. The consequence of this however is unknown.

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