

Investigations on *Trematomus bernacchii* immunoglobulins at gene and protein level

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Abstract: To study how teleosts have modified their immunoglobulin structures to adapt to the Antarctic environment, we investigated *Trematomus bernacchii* IgM at protein and gene levels. Serum IgM was purified, characterized and quantified. The nucleotide and deduced amino acid sequences of IgL and IgH were determined. Upon comparison with other teleosts, *T. bernacchii* IgH showed a higher hydrophilic character and the presence of two remarkable insertions, probably increasing the flexibility of the molecule. To investigate the amplitude of the antibody repertoire, a *VH* library was constructed. The analysis of rearranged *VH/D/JH* transcripts revealed a high occurrence of the RGYW motif and a clear bias in the usage of serine codons. Diversity generated by insertions/deletions occurred more often than in other species. *VH* sequences fell into only two gene families referred to as Trbe *VH I* and Trbe *VH II*. The deduced amino acid sequence of the membrane region was found to be the longest and an F/Y substitution was observed at a highly conserved position. Models of the transmembrane helices of *T. bernacchii* and other teleost Ig were constructed by molecular dynamics. Finally, the analysis of the antibody specificity revealed the presence of antibodies specific to the proteins of nematode parasites.

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Introduction

Immunoglobulins (Ig) appeared at the same time as the emergence of jawed vertebrates from their ostracoderm ancestors (about 450 million years ago) and in the same period as the other elements defining the adaptive immunity (T-cell receptor, MHC products and the recombination enzymes RAGs) (Marchalonis *et al.* 2002). The basic structure consists of two heavy (IgH) and two light (IgL) chains covalently linked by disulphide bridges. The molecule is present in two forms: secreted and membrane-bound.

An important feature of the secreted form is its high segmental flexibility, exhibiting at least seven different movements of the domains (Roux *et al.* 1997, fig. 1). This mobility has important functional implications, allowing the simultaneous recognition of the antigens and the induction of the effector functions, such as complement activation and the cell receptor interactions (Burton 1990). The central role of the “hinge” region in allowing the flexibility of the entire molecule, has recently been confirmed by analyses of crystallographic structures (Saphire *et al.* 2002). The rotation of the *VL-VH* dimer in relation to the *CL-CH1* dimer over a range of about 50°, in a manner described as “elbow motion” (Gerstein *et al.* 1994, fig. 7), is particularly relevant.

The membrane-bound Ig form, generated by an alternative splicing of the C exons, is exposed on the cell surface of B lymphocytes in association with the $Ig\alpha$ - $Ig\beta$ heterodimer. The resulting antigen receptor complex (BCR)

is able to transduce the signal of the antigen-binding site occupancy when translocated into specific microdomains of the cell membrane, described as “lipid rafts” (Cheng *et al.* 2001).

Different vertebrate classes have evolved distinctive structural features, gene locus organization and methods of generating antibody diversity (Flajnik 2002). Teleost serum Ig is a tetramer, each subunit showing the classical pattern of two IgH and two IgL chains (Warr 1995). The IgH genes are in the “translocon” configuration, whereas those of IgL are in the “cluster” configuration. The teleost IgH is slightly shorter than that of mammals. The position of invariant cysteine and tryptophan residues is conserved. But the number and distribution of “extra cysteines”, involved in inter-IgH chain and inter-subunit linkages, as well as of putative *N*-glycosylation sites varies within the teleost group; IgH contains a higher proportion of carbohydrates than that of mammals. The teleost membrane-bound form is produced by a primary transcript processing which differs from the typical vertebrate one by excluding the entire CH4 exon (Wilson & Warr 1992).

Comparative investigations of mammalian and cold-adapted teleost IgM are of great interest because of their significance in evolutionary adaptation in biochemical, genetic and physiological terms. To gain more insight into Ig structure and function we chose *Trematomus bernacchii* (Boulenger, 1902) as the model species a perciform of Notothenioidei family. This is the most abundant fish species in the sea near Terra Nova Bay station and lives at

constant temperature of -1.86°C .

Investigations on several aspects of the *T. bernacchii* Ig molecule and the genes encoding it are reported here.

Results and discussion

Biochemical analysis

The IgM purification was performed in four steps: 50% saturated ammonium sulphate precipitation, ultrafiltration at 0.7 atm through an Amicon YM 100 membrane, FPLC on a Bio-Prep SE-1000/17 column, HiTrap NHS-activated column, coupled with rabbit antibodies specific for *T. bernacchii* immunoglobulin H chain (Pucci *et al.* 2003).

Trematopus bernacchii Ig concentration was determined in serum, bile and skin mucus by ELISA from a standard titration curve constructed using purified *T. bernacchii* IgM. Levels of serum IgM, determined in different specimens ranged from 1.0 to 4.6 mg ml⁻¹, with an average 2.7 mg ml⁻¹, corresponding to 9.6% of the mean total serum protein content. The average IgM content was 4.1 mg ml⁻¹ in the bile and 0.1 mg ml⁻¹ in the cutaneous mucus.

Purified serum IgM analysed by SDS-PAGE, under reducing conditions, gave two bands of 78 kDa (IgH) and 25 kDa (IgL) respectively, and, under non reducing conditions, a single band of 830 kDa. Separated IgH chains, eluted from gel, were shown to reaggregate in tetramers even in the absence of IgL chains. By isoelectrofocusing analysis, purified IgM were shown to give at least seven bands in the pH range 4.4–6.5. The sedimentation coefficient, determined by analytical ultracentrifugation was 15.4 S. Carbohydrate analysis, performed on the H and L chain blotted bands, revealed the presence of glycosyl residues in the H chain. The contribution of carbohydrates was evaluated to be 12.8% of the entire chain. Purified Igs were hydrolysed by *N*-endoglycosidase F under different conditions and at least four different hydrolytic sites were revealed (Pucci *et al.* 2003).

Antibody specificity

To investigate the antibody specificity of the immunoglobulin from plasma and secretions, protein extracts were prepared from the parasite nematodes *Contracaecum osculatum* (Krabbe, 1878) (Coscia & Oreste 1998) and *Pseudoterranova decipiens* (Rudolphi, 1802) (Coscia *et al.* 2000b). The extracts were proteins excreted and secreted from living larvae (ESP), surface-associated proteins obtained by mild extraction (SAP), and cuticular soluble proteins obtained by extraction under strong reducing conditions (CSP). Using different immunoassays, these three preparations were tested for their ability to bind Antarctic fish antibodies. As determined by ELISA, the specific antibody binding activity was higher in SAP than in CSP. As determined by dot-blot immunoassay, the specific

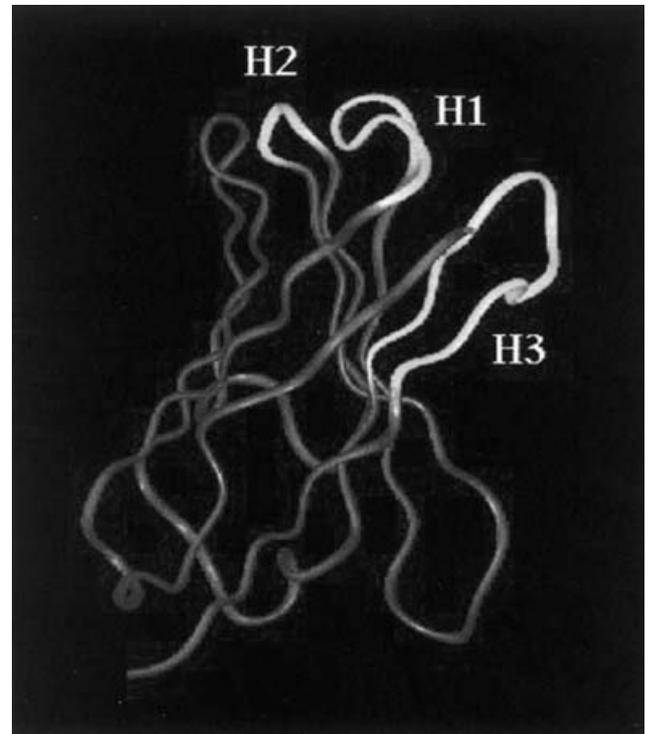


Fig. 1. Molecular model of *Trematopus bernacchii* Ig VH domain.

The loops H1, H2, and H3, correspond to the Complementary Determining Regions. The remaining part of the domain corresponds to the Framework Regions.

SAP binding activity was higher in bile than in plasma. A different number of antigenic components of SAP and/or ESP was identified by immunoblotting performed with plasma or bile antibodies. The *P. decipiens* 40 kDa antigen corresponded to the nematode haemoglobin; the *C. osculatum* 35 kDa antigen was identified as the nematode Glyceraldehyde 3-Phosphate Dehydrogenase. These results

Table I. Comparison of the character of *Trematopus bernacchii* VH domain amino acid residues with that of the consensus.

Kabat site	strand/loop	Character conservation (%)	
		Kabat database	<i>T. bernacchii</i>
12	A'3	b (64)	s (86)
13	A'4	n (51)	s (100)
18	B2	b (85)	n (85)
19	B3	n (55)	s (79)
23	B7	s (56)	n (85)
71	D6	n (48)	s (80)
73	D/E1	n (61)	s (89)
77	E1	n (54)	s (61)
78	E2	b (52)	s (82)
79	E3	n (89)	s (89)
82a	E7	n (69)	s (75)
83	E/F3	s (70)	n (77)
85	E/5	s (89)	b (71)
107	G2	s (46)	b (93)

The hydrophobic, neutral and hydrophilic residue character is indicated with b, n and s, respectively.

	VH ↓ CH1
<i>Heterodontus francisci</i>	QGTMTVTAAT--PSPPTLYGLCSCEQPNT
<i>Raja erinacea</i>	GGTMVTVTSAA--PSAPSPFLLFTCEDQGS
<i>Paralichthys olivaceus</i>	KGTMVTVTSAT--PAIPTLFFVMPCCSGSGTG
<i>Gadus morhua</i>	KGTVVTVSDSA--VQAPTVPFLVQCGSGTE
<i>Oncorhynchus mykiss</i>	KGTMVTVTSAS--STAPTFLPLAQCGSGTG
<i>Salmo salar</i>	KGTMVTVTSAS--STAPTFLPLAQCGSGTG
<i>Elops saurus</i>	RGTMVTVTTGE--QASPTVFPLVSCGA-TS
<i>Trematomus bernacchii</i>	KGTMVTVTSATATVSGPTVFPLMQCGSGTG
<i>Anarhichas minor</i>	KGTMVTVTSVT--SAAPTVPFLMQCGSGTG
<i>Amia calva</i>	KGTVTVTSAA--ATAPTFLPLVPCGSGAS
<i>Homo sapiens</i>	RGTLVTVSSGS--ASAPTFLPLVSCENSPS
<i>Mus musculus</i>	QGTSVTVSSES--QSFNVFPLVSCESPLS

Fig. 2. Sequence alignment of the VH-CH1 boundary of different actinopterygean and mammalian species. The arrow indicates the beginning of the CH1 domain.

lead to the conclusion that *T. bernacchii* parasitism by nematodes induces plasma and bile anti-parasite antibodies.

Gene cloning

To investigate IgH and IgL chain genes, cDNA was synthesized from poly(A)⁺ mRNA isolated from the spleen. The double-stranded cDNA fragments were ligated into the ZAP Expression vector. The cDNA expression library was then immunoscreened using rabbit IgG anti-*T. bernacchii* IgH or IgL chain. Immunopositive clones were isolated and sequenced (Coscia *et al.* 2000c).

To sequence as many VH gene segments as possible, a VH library was constructed from spleen RNA by anchored PCR (5' RACE), using an oligonucleotide primer complementary to the *T. bernacchii* CH1 exon. 5' RACE products were cloned into pCRII-TOPO vector and sequenced.

To sequence the transmembrane exons, total RNA was isolated from the headkidney and first strand cDNA was synthesized using an oligo (dT) primer linked to an appropriate adapter. Double-stranded cDNA was amplified using a 5' primer complementary to CH3 exon and a 3' primer complementary to the adapter. PCR products were cloned into pCRII-TOPO vector and sequenced.

IgH chain

The IgH amino acid sequence, deduced from the nucleotide sequence and submitted to GeneBank under the accession number AF094531, was analysed to investigate specific features. An atomic model of the VH domain (Fig. 1) was constructed using the canonical structures method for the hypervariable loops of the antigen binding site (Al-Lazikani *et al.* 1997) and standard homology modelling techniques for the framework regions (Coscia *et al.* 2000c). When the character (hydrophilic, neutral or hydrophobic) of each amino acid residue of *T. bernacchii* VH sequence was compared to the VH consensus sequence determined by Chothia *et al.* (1998, table 1), 14 differences were found (Table I). Ten out of 14 positions carried a hydrophilic amino acid residue versus a hydrophobic or neutral residue in the consensus. In the VH domain atomic model the side chain of all these hydrophilic residues was “exposed”, suggesting that increased interactions with the solvent would be needed to ensure solubility at low temperature.

A comparative analysis with the sequences of the Ig constant domains of other teleosts showed the presence of two remarkable insertions. The first one, presumably due to

	CH2] {	CH3
<i>Heterodontus francisci</i>	CQVSHQGVTVQSRNITGSQ-----	VPCSCNDPVIKLLPPSIEQVLLLEATVTLTC	
<i>Ginglymostema cirratum</i>	CQVKYKEVIQSWNITGPQ-----	YVSECHGTAKILPPPVEQVLLLEATVTLTC	
<i>Raja erinacea</i>	CQVNHEKDLKSNISMP-----	VSCDKLSITILPPQVEQELMEMTFTLTC	
<i>Carcharinus plumbeus</i>	CQVTHAPSNFNQGISLRYQ-----	EMTVLIRNPSIREVWTKTATLEEC	
<i>Hydrolagus collieii</i>	CQVTHVPTQTIIIVKNITKSS-----	CVDHGDVKVFLPPSQKQVLMESSVILTC	
<i>Xenopus laevis</i>	CVVEHAESGSLQEKNMKSILMCDT-----	PITPTSIQVITIPPSLESIFEKKSATLTC	
<i>Gallus domesticus</i>	CVVEGEMRNTSKRMECG-----	LEPVVQQDIAIRVITPSEVDIFISKSATLTC	
<i>Mus musculus</i>	CRVDHRGLTFLKNVSSSTCAA-----	SPSTDILTFTIPPSFADIFLSKSANLTC	
<i>Homo sapiens</i>	CRVDHRGLTFQONASSMVCV-----	DQDTAIRVFAIPPSFASIFLTKSTKLTC	
<i>Ictalurus punctatus</i>	CEFQQKNHNVFKEASYS-----	PGDTKQPQVKITGPSTEDILIKRAGQLEEC	
<i>Gadus morhua</i>	CRFANGKEPVD AHLTYGGGCD-----	EPSTKLEIDILPISLETMYLENNADLVC	
<i>Salvelinus alpinus</i>	CVFKNNAGNVRRTVGYTSSD-----	GPLHEHSVVINITPPSLEDMLMNKKAELVC	
<i>Salmo trutta</i>	CVFENKAGNVRRTVGYTSSD-----	AGPVHVHSVVINITPPSLEDMLMNKKAELVC	
<i>Elops saurus</i>	CQFIQEGEITEQTVKYSSAEC-----	PEAQIDAKISPPTEELFLOQTRTLTC	
<i>Trematomus bernacchii</i>	CLFEGKGEKGPSTVNRSLNITKCYDKPGEPIGSTDCPIADADVHI E PSMEDMFLQDKGTLYVC		
<i>Cyprinus carpio</i>	CEFVHKTGKKVIVA EYAVP-----	IQDCTNIAVDIVPPSLEDMLKNRQGVILKC	
<i>Danio rerio</i>	CVFEHNKRND SREIQYKDTMQ-----	DCIDDNVHIDIIPPTPEDMLKNRKGILKC	
<i>Amia calva</i>	CEVHHTEGWSKLNKNTSFIGECD-----	PLTTDVTVTIQGPEAKEVFLQKRGTLTC	
<i>Lepidosteus osseus</i>	CEFVHKTG SVLKNITYTSRQ-----	ETVKVVI E PPTNEEQFVKKTATLTC	
<i>Acipenser baeri</i>	CEVTFGSAKV FRTINSTSEPGD-----	CPANVHVGISPPSVEE I FLRKEATLTC	
strand	F [-----G-----]	[-----A-----]	[---B---

Fig. 3. Multiple alignment of the sequenceregion between the last conserved cysteine residue of the CH2 domain and the first one of the CH3 domain of different vertebrate species. Putative N-glycosylation sites and extra cysteines are underlined. β-strands are indicated below.

strand	F [-----G-----]	[----A----]	[---B-
	extracellular spacer	transmembrane domain	cyt
		4...8...11...15.18...23	
<i>Trematomus bernacchii</i>	SSQRIIDKEVGDNKGSTEVEEDD	MKSTAIT F IFLFLITLLFTIG T TAF	KVK
<i>Salmo salar</i>	----CLVLTDCPCSNTIETDRDS	MGKTA F T F II L FLITLLYGVGATAI	KVK
<i>Ictalurus punctatus</i>	-----CIWSTEIFHYEMEMDDDN	MANTAL T F V FLFLIT L FYSIG V T V F	KVK
<i>Gadus morhua</i>	---AFLVLTESQWSNAVDGQQDS	MQSTLN T F I IL F LIT L VYSIG T TAI	KIR
<i>Oncorhynchus mykiss</i>	---DCLVLTDCPCSNTMETDRD S	MGKTA F T F II L FLITLLYGVGATAI	KVK
<i>Cyprinus carpio</i>	---AVIVWLEHPLYEASDTDDSG	IANTAI T F V FLFLIT L FYSIG A T F V	KVK
<i>Danio rerio</i>	---AWIVWIEHPLFEPINADDSG	IANTAV T F I FLFLIT L FYSIG A T F V	KVK
<i>Acipenser baeri</i>	---AFLVLTESQWSNAVEYNGDS	LWNT V C T F I FLFLIT L FYSIG A T V T	KVK
<i>Homo sapiens</i>	---EGEVSAD----E--EG-FEN	LWATA S T F IV L FL L SL F Y S T T V T LF	KVK
<i>Xenopus laevis</i>	---AKVLFVD----EG-EE-MAS	LW T T A S T F I V L FL L SL F Y S A T V T LF	KVK
<i>Raja erinacea</i>	---PLPDYADNVPLEDYEDDRDN	I W T T A S T F II L FL L S I S Y GA V T L V	KVK

Fig. 4. Multiple alignment of the amino acid sequences of the *T. bernacchii* IgM membrane domains with those from other vertebrates. The CART motif residues are in bold.

a six nucleotide duplication, occurred at the VH-CH1 boundary (Fig. 2) affecting the “elbow motion” of the antigen combining region and not allowing hydrophobic interactions between the conserved phenylalanine and proline in the CH1 and the conserved leucine and isoleucines in the VH (Coscia *et al.* 2000c).

A second insertion was found in the region proposed to act as a “hinge”, at the CH2-CH3 boundary, and resulting in a spacer peptide longer than any other IgM hinge (Fig. 3) (Coscia *et al.* 2000a, p. 75). Evidence that the peptide is the product of a longer CH2 exon came from the genomic sequence of the CH2-CH3 intron. This peptide contained many charged residues and the presence of two putative *N*-glycosylation sites in close proximity which would suggest a role in protecting the exposed spacer from protease attacks. An unusually long hinge presumably ensures a wide internal movement of the Ig molecule.

Membrane-bound IgH

The sequence of the *T. bernacchii* membrane-bound Ig form was determined by amplifying headkidney RNA or testis DNA in RT-PCR performed utilizing an oligonucleotide complementary to CH3 exon as a 5' primer. The donor and acceptor splicing sites were identified in the genomic sequence. The deduced amino acid sequence of the membrane region was aligned with that from other species by the CLUSTALX programme (Fig. 4). The putative extracellular spacer, transmembrane, and cytoplasmic regions were defined utilizing the TM PRED programme. The *T. bernacchii* extracellular spacer was found to be the longest and the most hydrophilic; it contained also a putative *N*-glycosylation site.

A non-synonymous substitution occurred in the codon encoding the highly conserved Y18 residue, within the CART (Conserved Antigen Receptor Transmembrane) motif (Campbell *et al.* 1994). It is to be noted that in mammals an F/Y mutant was non-functional failing to

target antigens for processing (Shaw *et al.* 1990, 387–388, Cheng *et al.* 2001, 3694–3698). The different lipid membrane context between cold-adapted and homoeothermic vertebrates could account for the different behaviour. To investigate this point, models of the transmembrane helix of *T. bernacchii* and other teleost Ig were constructed by molecular dynamics and the orientation

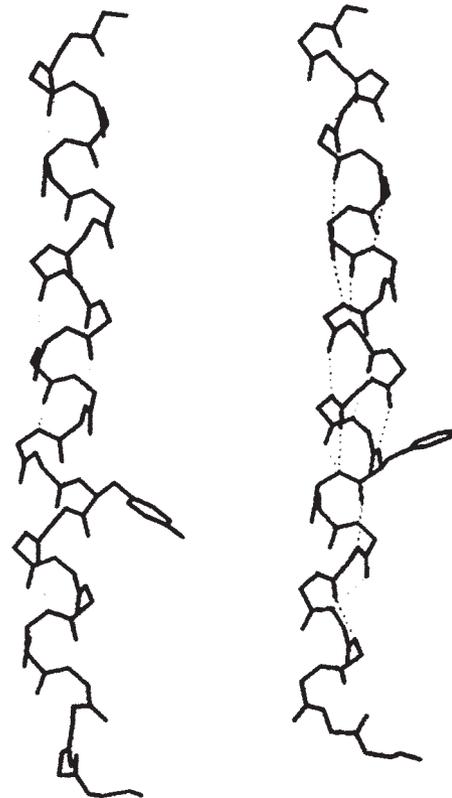


Fig. 5. Models of the transmembrane α -helix of *Trematomus bernacchii* (right) and *Gadus morhua* (left) constructed by molecular dynamics. The side chain of the residue at position 18 is shown. The C-terminus is at the bottom of the backbone.

of the *T. bernacchii* F18 residue was found to be 180° divergent from the Y18 of the other teleosts (Fig. 5), suggesting different interaction with different membrane lipids (unpublished data).

IgL chains

By sequencing clones isolated from a cDNA library immunoscreened with rabbit IgG specific for *T. bernacchii* IgL, two different isotypes were identified and referred to as TrbeCL1 and TrbeCL3. TrbeCL1 and TrbeCL3 shared 49.5% of the nucleotide identity and 36.5% of the amino acid identity. The percent of amino acid identity between TrbeCL1 and other teleost CL1 ranged between 58.5 and 91.7%, that between TrbeCL3 and other teleost CL3 ranged between 52.9 and 64.4% (unpublished data).

Diversity of the VH domain

To investigate the diversity of the immunoglobulin heavy chain variable domain, 45 clones containing complete or partial sequences of rearranged *VH/D/JH* segments were sequenced from the IgVH library (Coscia & Oreste 2003). *VH* sequences shared, on average, 79.9% nucleotide identity and defined only two gene families referred to as *Trbe VH I* and *Trbe VH II*, the latter comprising 89% of the *VH* sequences analysed in this study. A Southern blot analysis, performed with family specific probes, revealed that there are at least 25 genomic *VH* genes. A phylogenetic tree for *VH* gene families from various organisms, previously classified into eight groups, A-H (Ota *et al.* 2000, fig. 3), showed that *Trbe VH I* clustered with *VH* genes belonging to group D and *Trbe VH II* with those of group C. Four putative distinct D segments were found to contribute to the diversity of the third Complementary Determining Region (CDR), which showed a high glycine content. The Shannon entropy analysis (Stewart *et al.* 1997) revealed that the Framework Regions (FR) are very highly conserved. Of CDRs, CDR2 exhibited a mean entropy value higher than CDR1, contributing to variability in a significant manner. Moreover, eight distinct *JH* segments were identified. These findings suggest that diversity of *VH* genes may be limited in the Antarctic teleost *T. bernacchii* (Coscia & Oreste 2003).

Specific features of VH genes

Analysis of *VH* sequences revealed specific features (Oreste & Coscia 2002). The A+T content (58.6%) of the coding regions was found to be higher than that of other *T. bernacchii* sequenced genes (48.1%). Tandem repeats (direct or inverted) up to 8 bp and palindromic sequences were widely present in the hypervariable CDR1 and CDR2 regions. An unusual starting codon motif, AUGAUG, was present in the majority of the transcripts. The occurrence of

the RGYW motif (Purine-Guanine-Pyrimidine-Adenine or Thymine), identified as the preferential site of mutations (Foster *et al.* 1999), was examined. The frequency of the RGYW motif in the *VH* gene segments was 4.4, that is 1.4-fold higher than the expected frequency (0.3), and higher than the frequency (1.8) calculated for the entire constant region. When CDRs and FRs were analysed separately, the frequency was 2.4 in FRs and 5.9 in CDRs. The occurrence of the mutational motif in *T. bernacchii VH* segments was higher than that calculated in *Oncorhynchus mykiss VH* segments.

In addition, the usage of serine codons showed a clear bias for AGY (where Y is C/T) in CDRs and for TCN (where N is A/T/C/G) in FRs. Notably, the usage of the AGY codon in *T. bernacchii* is higher in CDRs and lower in FRs compared to *Salmo salar*.

Diversity generated by insertions/deletions occurred more often than in other species and essentially in CDRs; inserted bases were often repeats of adjacent bases, occurring at positions where a single-stranded DNA folding programme predicted a stem/loop transition. Alignment of *VH* sequences indicated that the gene conversion mechanism may contribute to generating diversity. This data suggests that the hypermutational events may also occur in teleosts.

Alignment of all the analysed sequences revealed that the same *VH* gene segment was present in four different *VH/D/JH* rearrangements, indicating that a given *VH* segment may be utilized more frequently than others. Six clones bearing the same *VH/D/JH* rearrangement, except for very few nucleotide differences, were also found. This can be interpreted either as a result of somatic mutations or as a PCR error.

Conclusions

Immunoglobulins from the cold-adapted teleost *Trematomus bernacchii* show specific features such as high glycosylation, and high flexibility, which probably account for the evolutionary adaptation to the low temperature of the Southern Ocean. The replacement of a highly conserved amino acid residue in the transmembrane domain indicates that a different lipid context may be crucial in signal transduction through cold-adapted cell membranes.

At the gene level, variability and mutability have been found to be more concentrated in the CDRs compared to other species. This indicates that the diversity of the variable domain is restricted to the loops, whereas the whole scaffold of the domain is strictly dependent on the environmental constraints.

Finally, from a functional point of view, direct evidence that Antarctic fish antibodies are involved in the immune response against the parasitic nematodes has been provided.

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