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DNA methylation analysis uncovers a fine-tuned regulation of expression of the polymeric Ig receptor gene in the Antarctic teleost *Trematomus bernacchii*

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ABSTRACT

The polymeric immunoglobulin receptor (pIgR) is an evolutionary conserved transmembrane glycoprotein that mediates the transport of polymeric immunoglobulins across epithelial barriers, essential for protecting host mucosal surfaces from pathogens. Although the function of pIgR is well described in mammals and teleost fish, its gene structure and expression regulation yet remain elusive. Previously, we identified the gene encoding the pIgR in the cold-adapted teleost *Trematomus bernacchii*, belonging to the suborder Notothenioidei, a dominant component of Antarctic fish fauna. Comparative analysis of pIgR encoding sequences from different teleosts revealed structural features unique to Antarctic species. Among them, a CpG island was detected within the promoter region of the *pIgR* gene, where DNA methylation is known to occur and influence gene expression. In the present work, we report the presence of an additional CpG island located in the second intron, upstream of a DNA transposon motif. Since epigenetic regulation of genes from Antarctic fish is almost unexplored, we experimentally assessed the methylation status of both CpG islands in gills, gut and liver. The promoter CpG island showed varying methylation levels across different tissues, suggesting a regulation of the *pIgR* gene expression in a tissue-specific manner. Conversely, the intronic CpG island was highly methylated in all analyzed tissues, supporting the role of DNA methylation in controlling transposon activity. This study provides the first evidence of epigenetic modulation of an immune gene in an Antarctic teleost species, offering novel insights into gene regulation and adaptation to extreme cold environments.

1. Introduction

Antarctic teleosts of the suborder Notothenioidei (order Perciformes) strongly dominate fish diversity and abundance in the Southern Ocean [1]. Notothenioid species have been classified into eight families: five are mainly Antarctic, collectively referred to as the superfamily Cryonotothenioidea, and three are non-Antarctic families [2]. The latters are considered as the basal taxonomic branch, having diverged early from the main lineage and remained in sub-Antarctic temperate seawaters, after the climatic and geographic isolation of Antarctica [3].

Significant genome modification events have played a crucial role in the diversification of Notothenioidei, allowing a number of physiological innovations, such as the acquisition of antifreezing glycoproteins, the lack of the classical heat-shock response, balanced by high constitutive expression of heat-shock protein 70, typically expressed in permanent cold-stressed conditions [4]. An exceptional loss affecting the family Channichthyidae is the inability to synthesize haemoglobin due to a single deletion event in the β-globin gene, while truncated remnants of the α-globin gene are maintained in their genomes [5–7]. The evolutionary modifications occurred in the Antarctic fish genomes have affected also the immunoglobulin heavy chain gene (*IgH*) locus. Earlier studies identified *IgM* and *IgT* genes in multiple Antarctic fish species, disclosing unexpected complexity of the *IgH* gene locus, likely due to random duplication and deletion events causing exon

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gain or partial loss [8–11].

Recent studies uncovered remarkable structural features in the gene encoding the polymeric immunoglobulin receptor (pIgR) in the Antarctic teleost *T. bernacchii* [12]. This gene exhibits extraordinary long intronic regions, carrying motives typical of transposable elements (TEs), including DNA transposons, SINEs and LINEs. Interestingly, lengthening of introns is associated with the acquisition of additional transcriptional factor motifs, such as IL-10, IFN and IRF-3. Notably, a further regulatory element, a CpG island, was detected in its promoter region, a feature absent in temperate fish species and in non-Antarctic relatives [12]. CpG islands are DNA stretches with higher CpG density, typically located in vertebrate gene promoter regions [13]. Cytosines within CpG dinucleotides can be methylated, forming 5-methylcytosines, by DNA methyltransferases, a well-conserved family of enzymes. DNA methylation is a key epigenetic process that regulates several biological mechanisms, such as gene expression, DNA structure remodelling and control of TE activity [14,15]. DNA hypermethylation in promoters and regulatory sequences induces transcriptional repression, whereas hypomethylation promotes gene expression [15]. In teleost fish, methylation patterns have been shown to vary significantly across tissues, influencing developmental processes and shaping physiological and adaptive responses [16–18]. Despite growing interest, epigenetic modifications in teleosts remain an emerging field [19].

While the pIgR plays a well-established role in mucosal immunity [20], its epigenetic regulation in teleost fish is poorly understood. To date, no studies have examined CpG methylation patterns of immune-related genes in Antarctic fish, nor their potential tissue-specific variability, leaving a critical gap in understanding how epigenetic mechanisms contribute to immune adaptation in extreme environments.

The CpG islands identified in the *T. bernacchii pIgR* gene are thus particularly challenging for exploring the role of epigenetic regulation under extreme cold conditions. The present study addresses this knowledge gap by analyzing the methylation status of the CpG islands within the *pIgR* gene in key mucosal immune tissues, gills and gut, and in the liver, an immune relevant organ in fish.

2. Material and methods

2.1. Sampling T. bernacchii specimens

Four specimens of *T. bernacchii* (Nototheniidae family) (Table 1), were caught by using hooks in the vicinity of "Mario Zucchelli" Station, Terra Nova Bay, Ross Sea (at 74° 42'S, 164°07'E), during the XXVII and XXIX Italian Antarctic Expeditions (2011–2012 and 2013–2014, respectively). Tissues and lymphoid organ were collected and immediately frozen in liquid nitrogen. All procedures were performed in compliance with relevant laws and institutional guidelines and the appropriate institutional committees have approved them.

2.2. Gene expression analysis of pIgR

Total RNA was extracted from 50 mg each of gills, gut, liver and muscle tissues collected from four *T. bernacchii* specimens, using RiboEx kit (GeneAll), following the manufacturer's instructions. Tissues were homogenized with a Potter-Elvehjem glass-Teflon, RNA quality was assessed by 2 % agarose gel electrophoresis, and concentration was determined by measuring absorbance at 260 nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). To avoid DNA genomic contamination, RNA samples were treated with DNase I (Thermo Scientific). First-strand cDNA was synthesized from 2 μg of total RNA using the 5X All-In-One RT PCR MasterMix (Bio Basic), following the manufacturer's instructions. Quantitative PCR (qPCR) reaction consisted of 2 μl of cDNA diluted 1:10 and 5 μl of Green-2-Go qPCR MasterMix (Bio Basic) in a final volume of 10 μl, including primers at a final concentration of 0.3 μM (primer sequences are listed in Table 2), according to the manufacturer's protocol. Amplification was performed on a Light Cycler 480 system, under the following conditions: 95 °C for 5 min, followed by 45 cycles of 95 °C (15 s), 60 °C (1 min). A final dissociation step was performed to generate a melting curve in order to verify the amplification specificity. Relative expression of the *pIgR* gene was calculated using the $2^{-\Delta\Delta Cq}$ method, with β-actin as the housekeeper gene (Table 2) and the muscle tissue as the negative control. Comparisons between muscle and mucosal or lymphoid tissues were performed using a one-tailed paired Student's *t*-test. Data are presented as ± standard error of the mean (SEM), and differences were considered statistically significant at $p < 0.05$.

Table 1
List of *T. bernacchii* specimens classified by sex, weight and length.

Specimen	Sex	Weight (g)	Length (cm)
TB01	FEMALE	211.34	25.5
TB02	MALE	112.52	20.0
TB03	MALE	154.20	22.5
TB04	FEMALE	228.50	26.0

2.3. CpG island prediction and primer design for bisulfite-sequencing PCR

Search for potential CpG islands within the *T. bernacchii pIgR* gene sequence was performed by the bioinformatic tool MethPrimer [21], using parameters such as the size, GC content and the ratio of observed to expected CpG. Primers for bisulfite-sequencing PCR (BSP) were designed flanking the CpG island 1 and CpG island 2 (Table 2).

2.4. DNA extraction and bisulfite-sequencing assay

DNA was extracted using PureLink® Genomic DNA Mini Kit (Thermo Scientific), according to the manufacturer's protocol. One hundred-fifty mg of muscle, liver, gut and gills each from four *T. bernacchii* specimens, were homogenized by Potter-Elvehjem glass-Teflon. DNA quality and concentration were evaluated by 1 % agarose gel electrophoresis and by a NanoDrop 1000 Spectrophotometer (Thermo Scientific). To analyze DNA methylation patterns, 1 μg of extracted genomic DNA was subjected to bisulfite conversion by using Epitect Bisulfite Kit (Qiagen) and eluted in 40 μl of sterile water. The bisulfite-converted DNA was then amplified by PCR using BSP primers flanking CpG island targets (Table 2). PCR reaction was set up in a final volume of 25 μl using 5 μl of bisulfite modified genomic DNA, 2.5 μl of each primer (final concentration 1 μM), 0.5 μl of dNTP mix (0.2 μM), 2.5 μl 10X DreamTaq Buffer, and 0.5 μl of DreamTaq Hot Start DNA polymerase (Thermo Scientific), up to volume with H_2O. Thermal cycling conditions were: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for

Table 2
List of primers used for qPCR analysis and bisulfite sequencing.

Primer name	Sequence	Application
TbBACTfw	5' – CCCAGATCATGTTCGAGACC – 3′	qPCR analysis
TbBACTrev	5' – CATAGATGGGCACTGTGTGG – 3′	qPCR analysis
TbrtpIgRFwd	5' – AAGAAGTGGTGTCGGAGTGG – 3′	qPCR analysis
TbrtpIgR Rev	5' – ACCAGCCTGTATCCCTCATC – 3′	qPCR analysis
CpG island 1 pIgR Fwd	5′– GGAATTTTGGATTGGGTTTAGTT – 3′	Bisulfite sequencing
CpG island 1 pIgR Rev	5' – ACTACTAACCATACTACAACAATAAACAAC – 3′	Bisulfite sequencing
CpG island 2 pIgR Fwd	5' – TTTTTATTGATGGTATAGATGTAATTTGGT – 3′	Bisulfite sequencing
CpG island 2 pIgR Rev	5' – ACACTCCCTTAAAATAAAATAAAAAAAA – 3′	Bisulfite sequencing
M13uni-21	5' – TGTAAAACGACGGCCAGT – 3′	Cloning sequencing

1 min, with a final extension at 72 C for 15 min. PCR products were analyzed on a 1.7 % agarose gel electrophoresis, then purified by using Expin PCR Gel SV kit (GeneAll) and finally cloned into the pCR2.1 cloning vector using TA Cloning Kit (Invitrogen). Positive clones were selected by the blue/white method and sequenced on ABI PRISM 3100 automated sequencer (Eurofins Genomics Europe Sequencing GmbH). Sequencing of clones for each sample was performed by using M13 forward primer (Table 2). Sequencing chromatograms were visualized using the software Codon Code Aligner (version 12.0.1) and the nucleotide sequence similarity was assessed against the GenBank database using the BLAST program. To determine the methylation status of each cytosine, the resulting sequences were aligned using ClustalW [22] and methylated cytosines were distinguished from those unmethylated, the latters being converted to thymines after bisulfite treatment.

Data are presented as ± standard error of the mean (SEM). Comparisons were performed by using two-tailed paired Student's *t*-test, differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Methylation status of the CpG island in the promoter region of *T. bernacchii pIgR* gene

Differential expression of the *T. bernacchii pIgR* gene was previously observed in the mucosal tissues gut and gills, and in the liver, a secondary lymphoid organ, with the highest expression in gills and moderate in gut and liver [12]. This expression pattern was confirmed in the present study through qPCR analysis performed on the same tissue samples employed for subsequent DNA methylation analysis (Fig. 1). Based on these earlier findings, the *T. bernacchii* genome assembly [23] was previously searched for DNA regulatory elements using the UCSC Genome Browser. The presence of a CpG island was highlighted in the promoter region of the *pIgR* gene (NW_022987689.1; position: 11,764,973–11,765,182; emerald rockcod fTreBer1.1), absent in basal

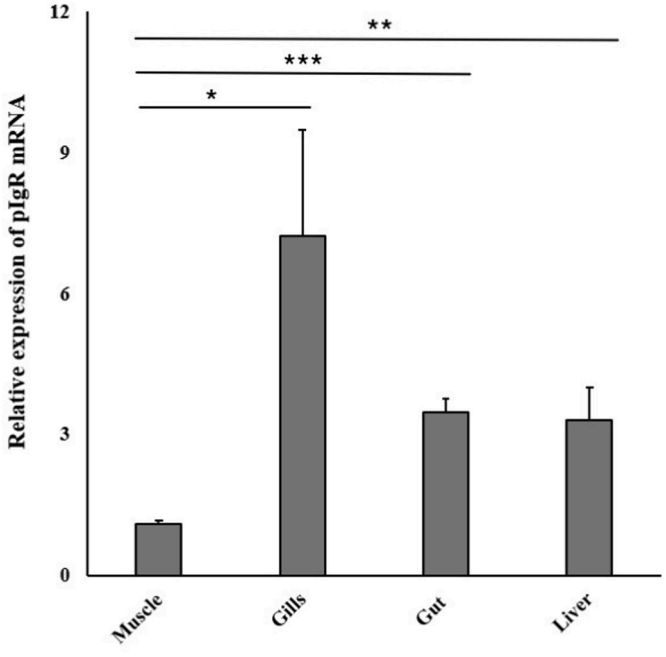

Fig. 1. Relative expression levels of *pIgR* in muscle, gills, gut and liver from *T. bernacchii* specimens. Data are presented as the mean gene expression relative to the housekeeper gene β-actin (±SEM). Muscle tissue was used as a negative control. Transcript levels were measured in duplicate from four *T. bernacchii* specimens (n = 4), in two independent experiments. Statistical significance was determined using a one-tailed Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

notothenioid species and in temperate Perciform counterparts [12]. This CpG island, thereafter referred to as CpG island 1, spans a region of 210 bp, consists of 21 CpG, and exhibits a GC content above 50 % with an ObsCpG/ExpCpG ratio above 60 % (Fig. 2).

In the present work, the methylation status of this CpG island was assessed to determine whether it accounts for tissue-specific regulation of *pIgR* gene expression. Region-specific bisulfite sequencing was performed on gills, gut and liver of *T. bernacchii*. Muscle was included as a negative control, being a non-immune tissue. The bisulfite-sequencing analysis of the 21 CpG sites within the CpG island 1 revealed tissue-specific methylation status consistent with the results of expression analyses (Fig. 3A). In detail, a methylation level of 81.9 % was observed in gills, which is 16 % lower than the level in muscle (97.6 %), 8 % than that in gut (89.3 %) and 6 % than that in liver (91.8 %) (Fig. 3B). Interestingly, comparative analysis of CpG methylation levels among tissues revealed that the first ten CpG sites were found to be differentially methylated, contrary to the remaining CpG sites [11–21], which fall in a conserved methylated-region (Fig. 3A). In detail, gills exhibited the lowest methylation level (69.2 %), corresponding to a decrease of around 30 % compared to muscle (96.2 %). Gut (79.5 %) and liver (86.6 %) showed a decrease of 17 % and 10 %, respectively (Fig. 3C). These results demonstrate that promoter CpG island 1 displays tissue-specific methylation differences, which likely contribute to the regulation of *pIgR* gene expression in mucosal and lymphoid tissues.

3.2. Methylation status of CpG island in the intronic region of *T. bernacchii pIgR* gene

To further characterize the *pIgR* gene locus, additional CpG islands were searched using the MethPrimer tool [21]. Another CpG island, referred to as CpG island 2, was identified in the second intron of the *T. bernacchii pIgR* gene (NW_022987689.1; position: 11,762,314–11,762,540; emerald rockcod fTreBer1.1), spanning about 200 bp and consisting of six CpG sites (Fig. 4A and B). In addition, CpG island 2 showed a GC content (>30.0 %) and ObsCpG/ExpCpG ratio (>0.6) similar to that of CpG island 1 (Fig. 4A and B). No additional CpG islands were detected within exons or in the remaining introns of the *pIgR* gene.

The bisulfite-sequencing analysis of CpG island 2 showed a high distribution of methylated CpG sites in all tissues analyzed (100 % in muscle, 94.8 % in gills, 96.8 % in gut and in liver) (Fig. 5A and B), suggesting the absence of tissue-specific methylation patterns observed for CpG island 1. Given that CpG island 2 is located approximately 150 bp upstream of a DNA transposon sequence, belonging to the PiggyBac family [23], this result is in line with the regulatory role of DNA methylation in the suppression of TE activity.

4. Discussion

The regulation of gene expression in teleost fish is fundamental for shaping physiological features in response to environmental and endogenous stimuli, enabling phenotypic plasticity across various habitats. Despite its importance, epigenetic mechanisms underlying gene regulation in teleosts have received limited attention [19]. Antarctic fish, inhabiting one of the most stable cold environments, provide a unique model to investigate how DNA methylation functions as an adaptive strategy for survival under constant sub-zero conditions.

A previous study on Antarctic fish reported a correlation between increased DNA methylation and progressive cooling of Antarctic seawaters [24]. Increased methylation likely enhances genome stability by repressing transposable elements and maintaining chromatin integrity in the Southern Ocean low-temperature environment. In addition, high methylation baseline may limit transcriptional flexibility, favouring a stable gene expression suited to constant environments [17]. In contrast, temperate fish display moderate global methylation and greater epigenetic plasticity, enabling dynamic gene regulation in response to variable conditions [25,26]. These observations suggest that highly stable

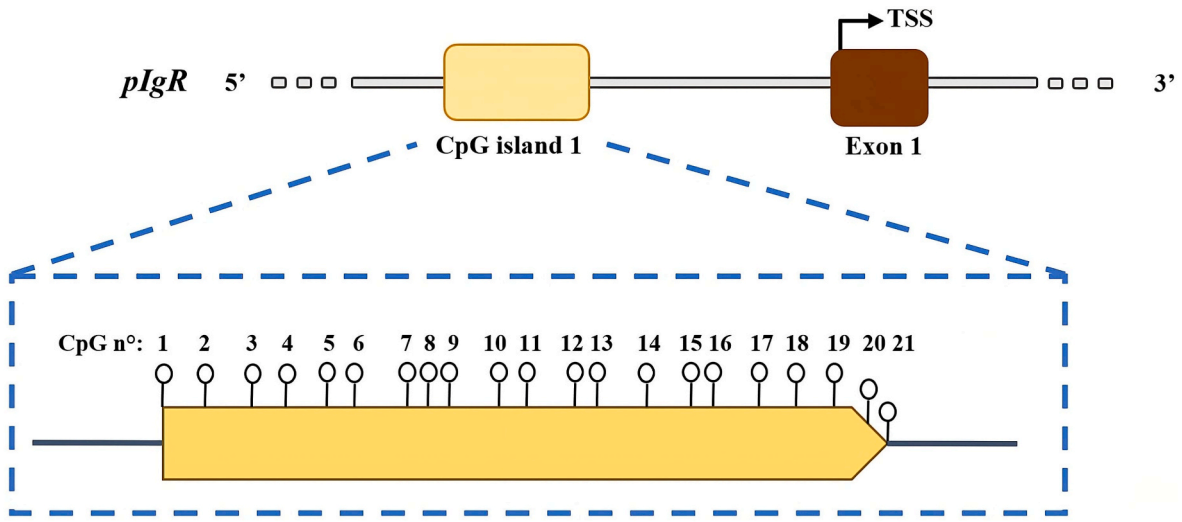

Fig. 2. Localization of CpG island 1 in the *T. bernacchii pIgR* gene locus. Schematic representation of partial *T. bernacchii pIgR* gene locus. Exon 1 is represented as a dark brown box and the transcriptional start site (TSS) is indicated by an arrow. CpG island 1 within the promoter region is represented as a yellow box. Details of the CpG island 1 structure are reported in the dashed blue rectangle at the bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

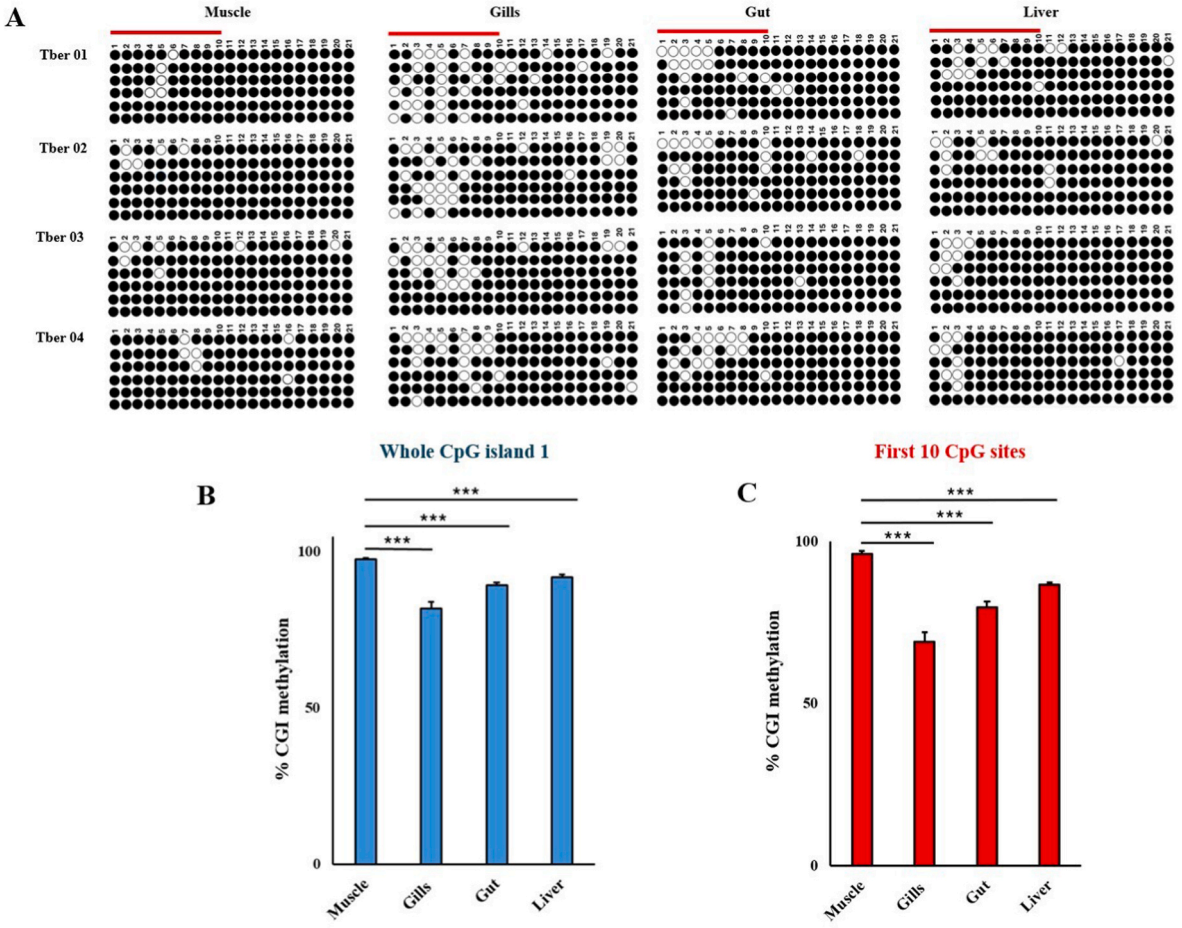

Fig. 3. Bisulfite-sequencing pattern of CpG island 1 in *T. bernacchii* muscle, gills, gut and liver. (**A**) Black filled circles represent methylated CpG sites; open circles indicate unmethylated CpG sites The position of the first ten CpG sites is marked by the red line. (**B**, **C**) Methylation levels of the whole CpG island 1 and of the first ten CpG sites in each tissue and organ on 24 clones derived from four individuals (n = 4). Data are presented as the mean percentage of methylation (±SEM). Statistical significance was determined using a two-tailed Student's *t*-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

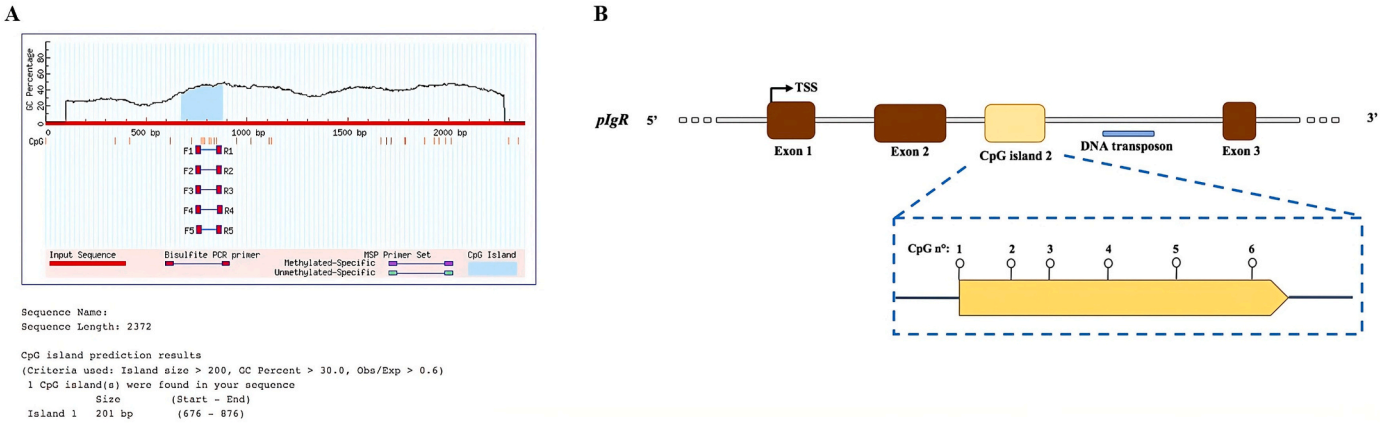

Fig. 4. Identification of CpG island 2 in the *T. bernacchii pIgR* gene locus. (**A**) MethPrimer analysis showing the detection of CpG island 2. (**B**) Schematic representation of partial *T. bernacchii pIgR* gene locus, encompassing the first three exons. Exons are represented as dark brown boxes and CpG island 2 is represented as a yellow box. The position of a DNA transposon motif is indicated by a light blue line. Details of the CpG island 2 structure are reported in the dashed blue rectangle at the bottom. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

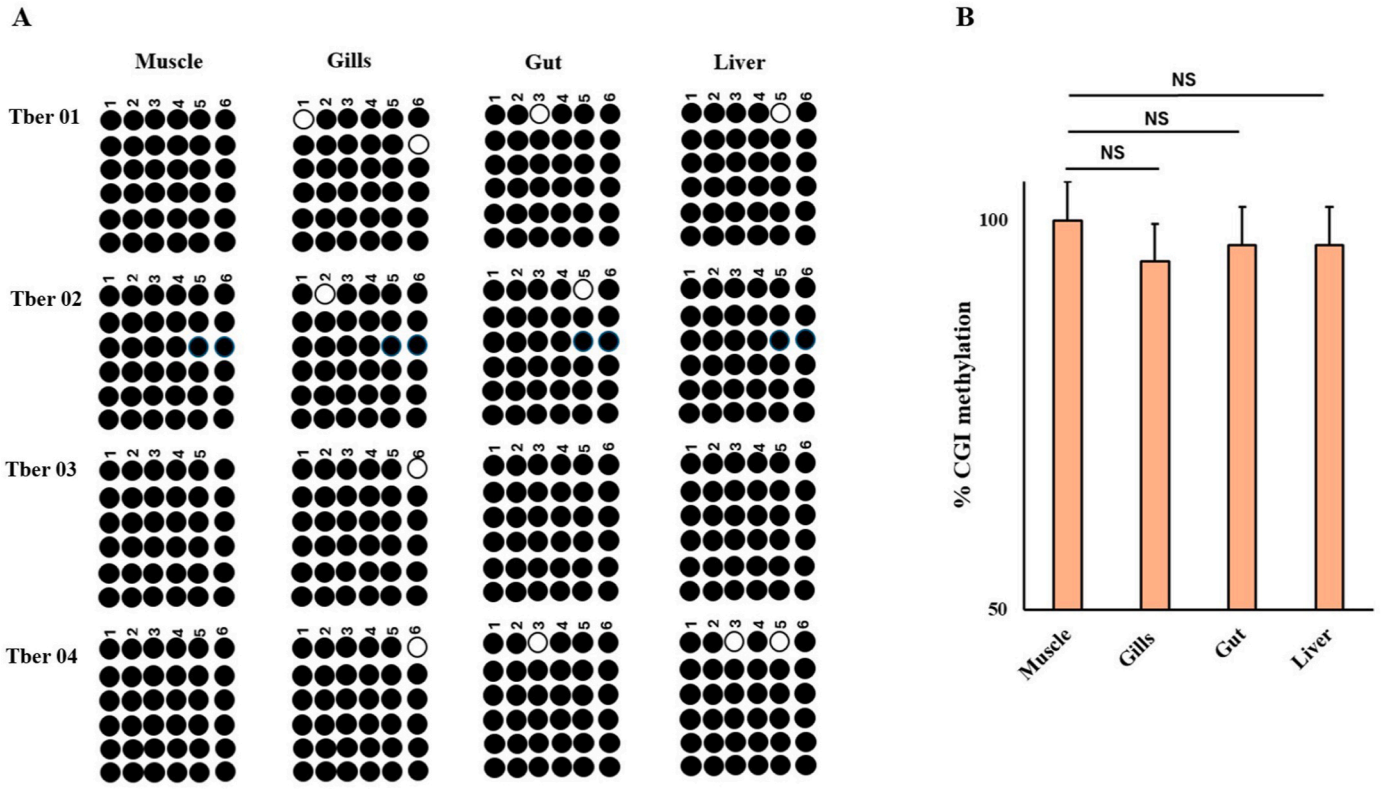

Fig. 5. Bisulfite-sequencing pattern of CpG island 2 in *T. bernacchii* muscle, gills, gut and liver. (**A**) Black filled circles represent methylated CpG sites; open circles indicate unmethylated CpG sites. (**B**) Methylation level of all six CpG sites in each tissue and organ on 24 clones derived from 4 individuals (n = 4). Data are presented as the mean percentage of methylation (±SEM). Statistical significance was determined using a two-tailed Student's *t*-test; results were not statistically significant (NS).

DNA methylation represents a key adaptive feature of Antarctic fish genomes.

Our findings reveal that, despite consistently high methylation (70–90 %) in CpG island 1 across tissues, minimal tissue-specific variations correlated with differential *pIgR* expression, highlighting its importance for fine-tuned regulation under cold conditions. The observed difference in methylation levels between gills and other tissues analyzed likely reflects their distinct biological role as a frontline barrier against pathogens in the aquatic environment [27]. The lower methylation level of the *pIgR* promoter in gills, associated with higher *pIgR* expression, suggests an epigenetic mechanism that enhances mucosal immunity under extreme cold. Notably, the high expression of *pIgR* in the gills of *T. bernacchii* is a natural phenomenon, in contrast with temperate fish where such upregulation is typically related to provoked infections [28]. In addition, the high *pIgR* gene expression observed in *T. bernacchii* gills correlates with increased expression of mucosal Igs levels (unpublished data), supporting the concept that a greater amount of pIgR is required for efficient Ig transcytosis across epithelial barriers [27,29]. This relationship underscores the functional significance of epigenetic regulation in maintaining mucosal homeostasis and host

protection in extreme environments.

Given the recognized key role of pIgR in polymeric Ig transport, further studies are needed to clarify how the methylation differences observed influence specific gene patterns and immune function. Future work could include functional validation, for example, using reporter gene assays to compare promoter activity between methylated and unmethylated CpG island 1 constructs.

Bisulfite sequencing, considered the "gold standard technique" for DNA methylation analysis [30], revealed that tissue-specific, differential methylation was confined to the first ten CpG sites within CpG island 1. This intriguing observation suggests a primary role for these sites in regulating *pIgR* expression across tissues, compared to the remaining CpG sites. Similar patterns have been reported in mammals, where only limited regions within CpG islands display differential methylation. These regions are thought to be important for tissue-specific gene regulation [31–33]. The proximity of these CpG sites to transcription factor binding motifs or cytokine-responsive elements likely contributes to their regulatory sensitivity. The methylation status of CpG sites can modulate the binding affinity of transcription factors that recognize CpG-containing DNA motifs [34]. We hypothesize that within the first ten CpG sites, tissue-specific methylation patterns may alter the accessibility and binding affinity of methylation-sensitive transcription factors, thereby influencing gene regulation.

Beyond its role in regulating gene expression, DNA methylation is a key process for controlling TE activity [35], which expanded significantly during Antarctic fish evolution and contributed to genomic modifications for adaptive radiation [4,23,36]. However, the molecular mechanisms involved in TE silencing remain poorly understood. In *T. bernacchii*, we identified a DNA transposon motif within the second intron of *pIgR* and retrotransposons, such as SINEs and LINEs, in the first and third introns [12]. Of note, a further CpG island, CpG island 2, located in the second intron, upstream of the TE motif, exhibited >90 % methylation across all tissues analyzed, suggesting a suppressive role in TE transcription. This result aligns with findings by Ohtani et al. [37], who reported that high CpG density with increased methylation degree in long terminal repeats, associated to endogenous retroviruses, mediates their suppression.

In conclusion, our study highlights the dynamic nature of DNA methylation and its potential role in shaping tissue-specific gene expression in a cold-adapted teleost fish. In particular, the identification of CpG islands with distinct methylation profiles provides new insight into how epigenetic mechanisms could modulate immune genes under extreme environmental conditions. These findings fill a current knowledge gap in comparative epigenetics and underscore the need for further research on the interplay between methylation patterns, immune function, and adaptation to cold ecosystems.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

CRediT authorship contribution statement

Alessia Ametrano: Conceptualization; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Salvatore Fioriniello:** Formal analysis; investigation; methodology; validation; writing – original draft; writing – review and editing. **Ennio Cocca:** Formal analysis; writing – review and editing. **Paola Italiani:** funding acquisition; project administration; resources; writing – review and editing. **Maria Rosaria Coscia:** Conceptualization; formal analysis; funding acquisition; methodology; project administration; resources; supervision; writing – original draft; writing – review and editing.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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