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# Helinoto, a Helitron2 transposon from the icefish *Chionodraco hamatus*, contains a region with three deubiquitinase-like domains that exhibit transcriptional activity

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ABSTRACT

Transposable elements have accompanied the evolution of the eukaryotic genome for millions of years. The recently discovered Helitron order (class II, subclass 2 single-strand DNA transposons) is common in eukaryotes and seems to play a highly active role in genome reshuffling. This study provides novel insights into the characteristics of Helinoto, a helitron isolated in the genome of the Antarctic fish *Chionodraco hamatus*. In particular, investigation of the structure of its 5′ and 3′ ends, which are involved in the transposition process, enabled identification of the characteristic motifs of the Helitron2 group. Moreover, identification of a deubiquitinating protease domain in the region upstream two consecutive OTU domains extended and strengthened the "deubiquitinase" character of the N-terminal portion of Helinoto. Finally, Helinoto transcriptional activity was detected in several *C. hamatus* tissues. Taken together, these data are particularly intriguing because they document high transcription levels for genes involved in ubiquitination, which ensures protein homeostasis in the extreme Antarctic environment.



## 1. Introduction

Transposable elements (TEs) have accompanied the evolution of the eukaryotic genome for millions of years. Such persistence suggests a strong influence of these elements on the evolutionary trajectory of their hosts. Especially in fish, the identification of an increasing number of TEs, some of which are still active (Kawakami and Shima, 1999; Böhne et al., 2012), is contributing to our understanding of genome rearrangements (Koga et al., 1996) and highlighting a growing number of cases where TE recruitment triggered new gene formation (Slotkin and Martienssen, 2007; Feschotte, 2008; Oliver and Greene, 2009).

Helitrons, a recently discovered order of rolling-circle (RC) subclass 2 DNA transposons (Wicker et al., 2007), are particularly widespread in eukaryotes and seem to have a key role in genome reshuffling (Lal et al., 2009; Barbaglia et al., 2012; Han et al., 2013). Unlike most other DNA transposons, which move in the genome via transposases, helitrons encode a protein with an RC initiator motif and a PIF1-like DNA helicase domain (Kapitonov and Jurka, 2007), and form their own order.

Because they replicate via an RC mechanism, helitrons present distinctive 5′ and 3′ terminal structures lacking the inverted repeats of class II elements, and transposition does not generate target site duplications. Until recently helitron ends were marked only by 5′ TC or 3′CTRR motifs (where R is a purine) and a short hairpin structure a few nucleotides upstream the 3′ end.

A certain variability in their size and copy number entails that helitrons are not easy to identify in genomes. As a result *in silico* analysis has become the most efficient approach to finding autonomous and non-autonomous representatives of this order of transposons.

In plants, where helitrons were initially identified by computational analysis, it has been shown that they often capture gene fragments, giving rise to a chimeric linkage between gene sequences originally located in distant chromosomal regions or separated from each other (Du et al., 2009). Helitrons have been shown to play a very active part in plant gene reshuffling; in maize at least 20,000 gene fragments are estimated to have been captured and shuffled by helitrons (Feschotte and Pritham, 2009; Yang and Bennetzen, 2009a).

In fish helitrons belonging to different subfamilies have been found to accumulate differentially in a number of teleost genomes. Notably, helitrons isolated in *Danio rerio* (Poulter et al., 2003), *Xiphophorus maculatus* and different poeciliids (Zhou et al., 2006) seem to have preserved their structure. Fish helitrons are characterized by an apurinic–

*Abbreviations:* ATIR, short asymmetrical inverted terminal repeat; DUB, deubiquitinase; ENDO, endonuclease domain; HEL, helicase domain; ORF, open reading frame; OTU, ovarian tumor protein domain; RC, rolling-circle; REP, replication initiator protein domain; SRA, sequence read archive; UPS, ubiquitin proteasome system; USP, ubiquitin-specific protease.

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apyrimidinic-like endonuclease at the C-terminal, probably captured from a non-long terminal repeat retrotransposon, whereas a cysteine protease domain related to Drosophila ovarian tumor (OTU) protein is located in the N-terminal part of the sequence (and appears duplicated in some elements). Putative endonucleases and proteases have been acquired sequentially at least 600 million years ago, and due to selective pressure they are still functional in elements from sea urchin, lancelet and teleost fish (Zhou et al., 2006). In fact, the enzymatic activity of the endonuclease domain can modulate fragment excision and integration processes, whereas the OTU1 and OTU2 domains may be involved in processing the large helitron precursor proteins (REP, HEL, ENDO and OTU). These domains may participate in degradation of REP/HEL protein through the ubiquitin (Ub) pathway (Balakirev et al., 2003) or activate a DNA repair pathway after or during transposition, to minimize host genome damage (Bao et al., 2010).

We previously reported the isolation of an almost intact 9 kb helitron, Helinoto (DHx_*Helinoto*_ GU014476), from the genome of the Antarctic ice fish *Chionodraco hamatus* (Cocca et al., 2011). Helinoto has all the characteristic domains encoded by the major autonomous helitron elements, i.e. REP, HEL, a C-terminal apurinic–apyrimidinic-like endonuclease, and two OTUs in the N-terminal part of the sequence. The presence and distribution of helitrons seem to be independent of climate and of the environmental conditions of their hosts. However, hybridization analysis with Helinoto probes suggested a fairly common presence of the transposon in the genome of Notothenioidei, whose life in a stable, extremely cold environment seems to have induced distinctive adaptations of several biochemical and physiological functions. Notothenioids are considered a monophyletic group and comprise eight families, with the Bovichtidae family occupying the first diverging position and Channichthyidae the most derived position of the suborder (Eastman, 2005).

This study: i) characterized the 5′ and 3′ ends of Helinoto and identified distinctive motifs of the Helitron2 group; ii) found a deubiquitinating protease domain in the region upstream the two OTU domains, thus extending and strengthening the "DUB" (DeUBiquitinase) character of the N-terminal portion of Helinoto; and iii) provides evidence for the transcriptional activity of Helinoto in several *C. hamatus* tissues.

## 2. Material and methods

### 2.1. Ethical procedures

The sample collection and animal research conducted in this study comply with Italy's Ministry of Education, University and Research regulations concerning activities and environmental protection in Antarctica and with the Protocol on Environmental Protection to the Antarctic Treaty, Annex II, Art. 3.

### 2.2. Animal sampling

Adult *C. hamatus* specimens were collected in the vicinity of Mario Zucchelli Station (MZS) on Terra Nova Bay coast (74°42′S, 164°07′E) during the 25th and 27th Italian Antarctic expeditions (2009–2012). Specimens were kept in running seawater at −2 to +1 °C until tissue sampling. They were killed by cutting the spinal cord. Tissues were dissected from specimens and immediately frozen. Tissues and cells were stored at −80 °C.

### 2.3. Amplification of 5′- and 3′-termini of Helinoto by inverse PCR

Inverse PCR is used to amplify unknown DNA flanking one end of a known DNA sequence (Ochman et al., 1988).

Starting from the known partial sequence of Helinoto (Cocca et al., 2011) we identified the restriction sites and designed the oligonucleotide primer pairs required for inverse PCR at its 5′ and 3′ terminal regions (Fig. 1). Genomic DNA from *C. hamatus* testis was obtained with the DNA Isolation Kit for Cells and Tissues (Roche, Germany). Aliquots of 5 μg of genomic DNA were digested with 20 U HindIII (5′-terminus) or BamHI (3′-terminus) in the appropriate buffers (mixture volume of 50 μl), purified using PCR clean-up kit (Macherey-Nagel, Germany) and then self-ligated using T4 DNA ligase (New England BioLabs, USA). Ligation reaction mixtures (volume of 20 μl), consisting of 1× reaction buffer (ATP 1 mM, $MgCl_2$ 10 mM, DTT 10 mM and Tris–HCl 30 mM, pH 7.8), 400 U T4 DNA ligase and 1 μg of digested DNA, were incubated overnight at 16 °C. The primers designed for amplification are listed in Table 1. PCRs were carried out in 20 μl volume with 100 ng of ligated DNA, 0.5 μM primers, 200 μM dNTP, 3 mM $MgCl_2$, 1 U EuroTaq polymerase (EuroClone, UK), and appropriate reaction buffer. The following conditions were used: initial denaturation at 94 °C for 1 min followed by 30 cycles at 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 3 min ending with a single cycle of 7 min at 72 °C to ensure full extension of all products.

### 2.4. Cloning, screening and sequence analysis

The amplification mixtures were directly ligated into the pSC-A vector (StrataClone PCR Cloning Kit, Stratagene, USA), designed for TA cloning, and screened by colony PCR with the primer pairs used for the inverse PCR reactions. Clones were selected by the size of their inserts. Plasmid DNA minipreps (GenElute™ Five Minute Plasmid Miniprep Kit, Sigma) were sequenced in an ABI PRISM 3100 automated sequencer (PRIMM, Italy). The amplicons were analyzed using the CENSOR web server (http://www.girinst.org/censor/index.php), where users align query sequences against the Repbase database of repetitive DNA elements, which includes the Helinoto sequence.

The clones that were found to contain the Helinoto fragments predicted by the experimental design (i.e. corresponding to the regions next to the inverse PCR primers, see Fig. 1) were further analyzed using the CLC Main Workbench 6.9 program (CLC bio, 2013), which allows complete editing, alignment, and secondary structure prediction of nucleotide sequences.

To establish whether the construct identified by *in silico* analysis did represent the full-length structure of Helinoto, a long-range PCR was performed at 50 °C using primers H4B9lpcrFor and H4B9lpcrRev (Table 1) and Expand Long Range, dNTPack kit (Roche) according to the manufacturer's recommendations.

The new construct was deposited in the GenBank database; the sequence was updated under accession number GU014476.

#### 2.4.1. Phylogenetic analysis

Phylogenetic analysis of DUB domain amino acid (aa) sequences was performed by two approaches:

1) Maximum likelihood (ML) analysis using the MEGA program (v. 6, Tamura et al., 2013); workflow: Muscle algorithm alignment, alignment test by "Find Best DNA/Protein Models (ML)" option, ML analysis by "Construct/Test Maximum Likelihood Tree (ML)" option (OTU: substitution model LG + G, Gamma 5,46; USP: substitution model LG + G, Gamma 1,35), bootstrap test: 1000 replicates.
2) Bayesian analysis with MrBayes 3.1.2 program (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=mrbayes; Dereeper et al., 2008); workflow: Muscle algorithm alignment, ML models and parameters as for MEGA analysis (v. 6), 100,000 generations.

Phylogenetic trees with the best consensus were considered for subsequent analyses.

#### 2.4.2. FASTA

The aa sequence corresponding to the Helinoto open reading frame (ORF) was analyzed by Protein Similarity Search using the FASTA suite of programs [http://www.ebi.ac.uk/Tools/sss/fasta/, version: 36.3.5e Nov, 2012 (preload8), database: uniprotkb].

*2.4.3. Phyre²*

The webserver Phyre² — Protein Homology/analogY Recognition Engine V 2.0 (Kelley and Sternberg, 2009 — http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) was used for protein domain structure prediction (Kelley and Sternberg, 2009).

*2.4.4. NCBI/BLAST*

NCBI/BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted to compare the Helinoto sequence with seven transcriptomic libraries of notothenioids (SRX088548 and SRX089045 from the liver of *Notothenia coriiceps*, SRX089044 from the brain of *N. coriiceps*, SRX089046 and SRX089047, respectively from the brain and the liver of *Pleuragramma antarcticum*, and SRX089048 and SRX089049, respectively from the brain and the liver of *Chaenocephalus aceratus*).

*2.5. Trancriptional analysis*

Total RNA was isolated from a variety of *C. hamatus* tissues according to the NucleoSpin RNA II kit (Macherey-Nagel) protocol, with an on-column DNase I step. RNA concentrations were determined using a Qubit fluorometer (Invitrogen). RNAs were then reverse-transcribed using SuperScript VILO MasterMix (Invitrogen).

RT-PCR was performed using total RNA from the liver as a template and the following oligonucleotide pairs designed on the Helinoto sequence: Help3For/OTUdomRev, Help2For/OTUdomRev, and REPdomFor/HELdomRev (see Table 1 for sequence and $T_m$). Amplification was performed as follows: 94 °C for 2 min, 40 cycles of 94 °C (30 s), 65 °C (30 s), and 72 °C (2.5 min), with a final extension at 72 °C for 7 min. A parallel amplification was conducted with the same RT-PCR conditions but using *C. hamatus* genomic DNA as the template.

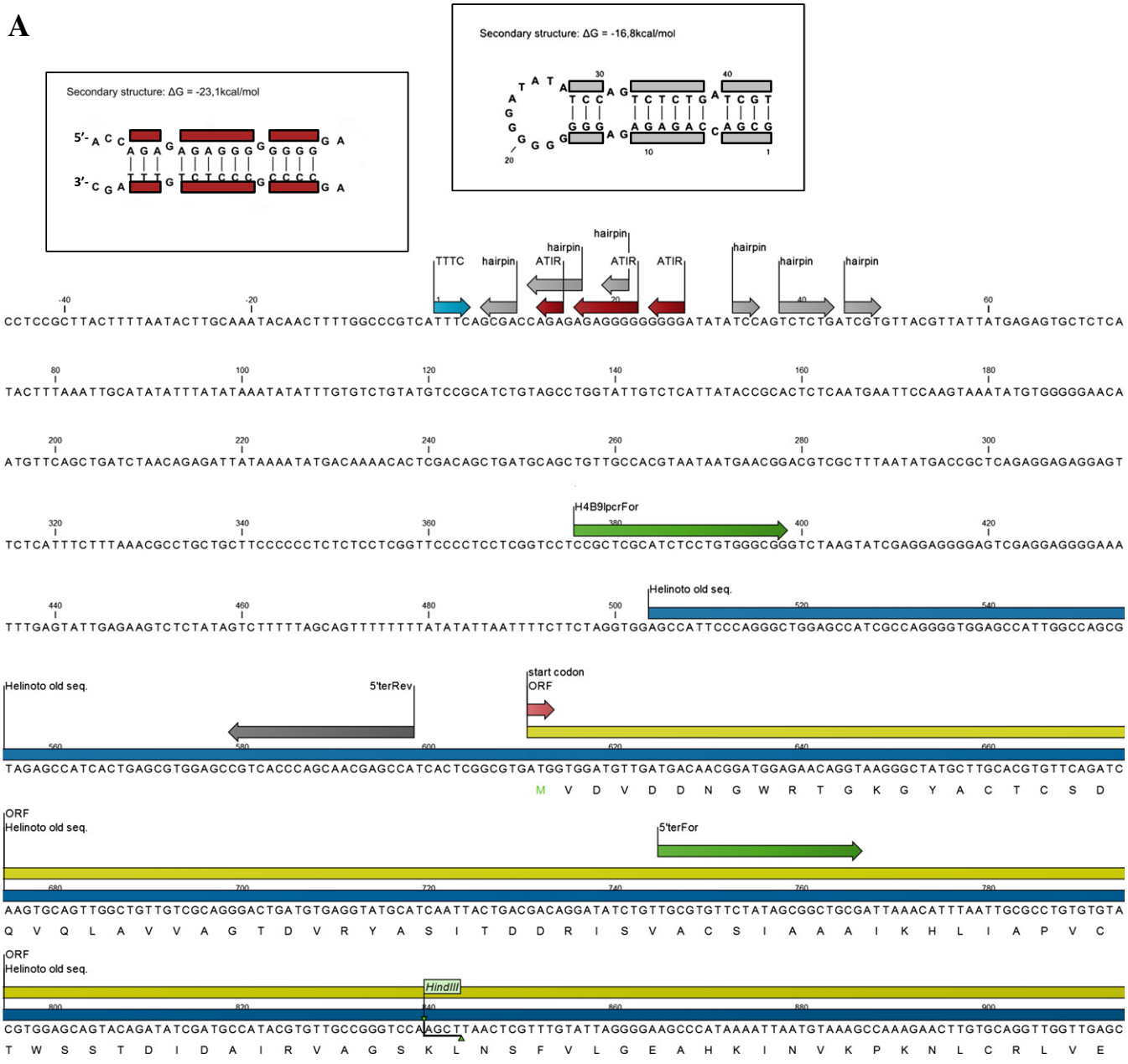


**Fig. 1.** H4–B9 Helinoto 5′- (A) and 3′-terminal (B) regions. Consensus motifs and sequence regions described in the text are highlighted by arrows. The primers and restriction sites utilized for inverse PCR procedure are also shown. The predicted hairpin structures and pairing of ATIR regions are boxed, with the indication of the associated ΔG (kcal/mol) values.

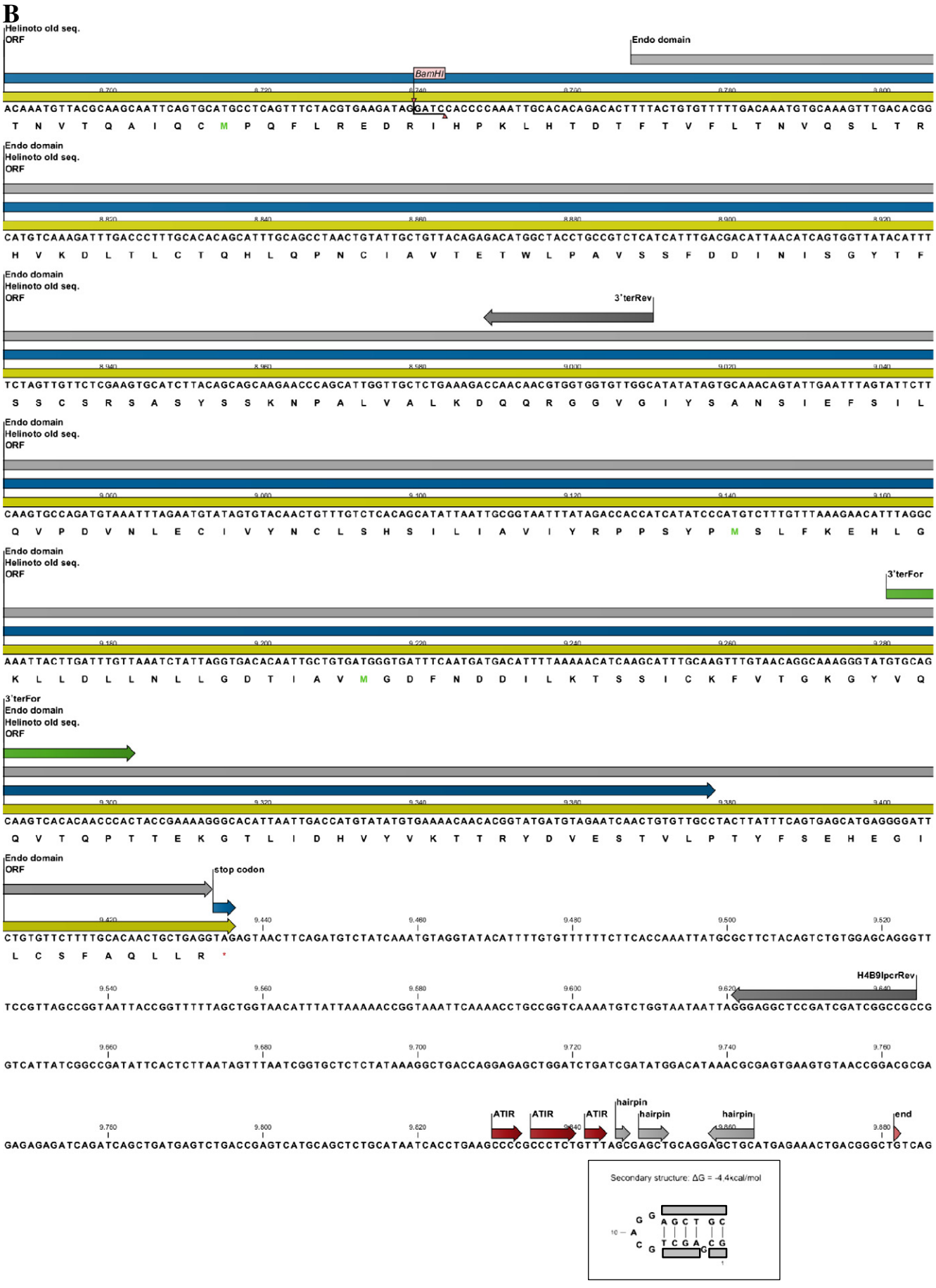


**Fig. 1** (*continued*).

**Table 1**
Sequence and $T_m$ of primers used for PCR experiments (see the Material and methods section).

| Primer | Sequence | $T_m$ |
|---|---|---|
| 5′terFor | TGCGTGTTCTATAGCGGCTGCG | 66 °C |
| 5′terRev | TGGCTCGTTGCTGGGTGACG | 65 °C |
| 3′terFor | GTGCAGCAAGTCACACAACCCAC | 66 °C |
| 3′terRev | GCCAACACCACCACGTTGTTGG | 66 °C |
| H4B9lpcrFor | CCGCTCGCATCTCCTGTGGGCGG | 73 °C |
| H4B9lpcrRev | GCGGCCGATCGATCGGAGCCTCCC | 70 °C |
| Help3For | CGACTGTATTGTAGTAGTTGATTGTGGCACAC | 66 °C |
| Help2For | ACAAACTGCTTTCGGTTCCAGATCTGC | 66 °C |
| OTUdomRev | ACCACGCGTTTGTTCCGCCA | 67 °C |
| REPdomFor | TGGCAGCGCGCACAGAATGA | 67 °C |
| HELdomRev | TTTCACTGGCGGCAGCTGGG | 67 °C |
| OTU1For | GGTTCATCAAATGCAGAGCA | 59 °C |
| OTU1Rev | TCTGACATGGAGGAGGTCAA | 59 °C |
| REPFor | GGTAGAACACAGACCGCTGAA | 60 °C |
| REPRev | CACAGGATTACGACGCAAGA | 59 °C |
| HELFor | CCCCATTTGGTAAAGTTAGTGTG | 59 °C |
| HELRev | CAGGGCATTTCCTTTCACTG | 60 °C |
| β-ACTINFor | ATGTTCGAGACCTTCAACACC | 59 °C |
| β-ACTINRev | CGACCAGAGGCGTACAGG | 60 °C |

The PCR products were then analyzed on 1% agarose gel, purified by StrataPrep DNA Gel Extraction Kit (Stratagene), and sequenced in an ABI PRISM 3100 automated sequencer (PRIMM).

*2.5.1. Quantitative RT-PCR*

To test primer efficacy, 100 ng of cDNA and its dilution series was amplified by quantitative real-time PCR (qPCR) on an iCycler iQTM system (Bio-Rad) using 300 nM gene-specific primers, Maxima H SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific), and the following PCR conditions: 1 cycle at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The level of expression of β-actin gene was used as an internal control for normalization. Raw cycle threshold (Ct) values obtained for "target" amplicons (OTU 1, REP, HEL) were compared with the Ct value obtained for β-actin transcripts ("ref" gene). The final graphic data were derived from the formula $R = (E_{target})^{\Delta Ct_{target}\ (control\ -\ sample)} / (E_{ref})^{\Delta Ct_{ref}\ (control\ -\ sample)}$ (Pfaffl, 2001). The Universal Probe Library Assay Design Center (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000) was used to design primers. The primer pairs used are listed in Table 1. As predicted from the Helinoto sequence amplicon sizes were: OTU 1, 63 nt; REP, 69 nt; and HEL, 74 nt; the size of the normalizing amplicon (β-actin) was 74 nt. All data are expressed as mean expression fold change; samples were run in triplicate.

## 3. Results

*3.1. Helinoto belongs to the Helitron2 group*

Helitrons have attracted strong interest ever since their discovery in 2001 (Kapitonov and Jurka, 2001). However, the lack of clear terminal markers considerably hampers their identification. Until recently helitrons have been identified by very short consensus sequences, i.e. "TC" at the 5′ terminus and "CTRR" at the 3′ terminus, with the latter being preceded by a small hairpin structure, and have preferentially been found in the "AT" dinucleotide (Yang and Bennetzen, 2009b). However, two recent studies have identified helitrons with new, different terminal markers, i.e. 5′ and 3′-subterminal inverted repeats (SIRs) in non-autonomous helitrons from the Lepidoptera genome (Coates et al., 2012) and short asymmetrical inverted terminal repeats (ATIRs), identifying helitrons belonging to a new phylogenetic group called Helitron2, in various eukaryotic genomes (Bao and Jurka, 2013). The fact that the Helinoto sequence previously characterized by our group, corresponding to an 8907 bp continuous ORF (Cocca et al., 2011), was incomplete at the terminal regions prompted us to use inverse PCR to extend our investigation. We isolated several positive clones belonging to two categories: H-type (with HindIII), containing fragments corresponding to the 5′ terminal, and B-type (with BamHI), containing fragments corresponding to the 3′ terminal. Although we obtained 16 H-type and 13 B-type positive clones, both clone sets belonged only to two subgroups, H4 and H22, and B4 and B9, respectively. Four different Helinoto molecules, H4–B4, H4–B9, H22–B4, and H22–B9, were obtained by trying all possible combinations of the fragments. The terminal regions of these potential sequences were then analyzed for the markers of this family of mobile elements.

*3.1.1. H4–B9 construct*

Results were satisfactory only for the H4–B9 construct. The presence of an ATIR at each terminal and of the other motifs described in Helitron2 elements identified Helinoto as a member of this group. To establish whether the H4–B9 construct represented the full-length structure of Helinoto we performed a long-range PCR, which yielded a genomic fragment of about 9.3 kb. Its length was consistent with the primers used (Supplementary file 1), which were designed on the 5′ and 3′ terminal regions obtained by inverse PCR (Fig. 1, A and B). Partial sequencing of the amplicon at both terminals confirmed that it corresponded to Helinoto (data not shown).

The H4–B9 construct (Fig. 1) had a total length of 9882 bp, because the two terminal fragments added 504 nt at the 5′ and 477 nt at the 3′ end. As a result the ORF of Helinoto measured 8826 bp, with the ATG start codon at positions 611–613 and the stop codon (TAG) at positions 9434–9436, corresponding to a 2941 aa polypeptide. The start codon was encompassed in the sequence published by our group (Cocca et al., 2011), whereas the new 3′ fragment completed the ENDO domain. The ATIRs covered 15 nt regions (AGAGAGAGGGGGGGG/CCCCGCCCTCTGTTT) at positions 12–26 (5′) and 9830–9844 (3′) that were not repeated elsewhere in the construct. The ATIR at the 5′ end was preceded by a TTTC tetranucleotide (positions 1–4) and was found on one of the two arms of a hairpin structure (positions 6–48, $\Delta G = -16.8$ kcal/mol) whereas, as commonly seen in this family of helitrons, the ATIR at the 3′ end preceded another hairpin structure (9846–9863, $\Delta G = -4.4$ kcal/mol) that was 19 bp away from the last predicted nucleotide (G). The entire helitron was inserted into the AT dinucleotide.

*3.2. Helinoto contains a "USP" domain*

After gathering information on the terminal regions of Helinoto and identifying the start and stop codons of its only, long ORF the protein sequence corresponding to the ORF translation (2941 aa) was analyzed. The Fast Family and Domain Prediction tool in the FASTA suite of programs recognized all the domains already described in Helinoto (starting from the 5′ terminus: OTU 1, OTU 2, REP, HEL, and ENDO) and added a new region corresponding to a PFAM domain called "Peptidase C76, herpes virus UL36 UL36 deubiquitinating peptidase (IPR006928)" (Supplementary file 2). This domain was not detected by FASTA analysis in the Helinoto sequence, probably due to low similarity in this region, but it was recognized in several polypeptides exhibiting a helitron-like overall structure (marked by a red arrow in Supplementary file 2). The sequences sharing the highest similarity with Helinoto were Q7M559_DANRE and Q7M560_DANRE from *D. rerio*, H3HEQ6_STRPU and H3J9Z2_STRPU from *Strongylocentrotus purpuratus*, H2MFR5_ORYLA and H2M5C2_ORYLA from *Oryzias latipes*, and G3Q6B8_GASAC and several others from *Gasterosteus aculeatus*, which are aquatic organisms (teleosts and sea urchin). Interestingly, the Peptidase C76 domain was found in the N-terminal region of all these polypeptides; in some it was followed by an OTU domain (reported as ovarian tumor, Otubain, and indicated by a yellow arrow in Supplementary file 2).

Further characterization of the Helinoto region corresponding to the two OTU domains and an additional segment at their N-terminal, which

seemed to contain the newly identified PFAM domain, was obtained by submitting its sequence to the web-based Phyre[2] server, where query sequences are compared to those of homologous proteins with known 3D structure. Phyre[2] recognized the structure of a Ub-specific protease encoded by murine cytomegalovirus tegument protein m48 (PDB Entry: 2j7q) right in the N-terminal portion of the submitted sequence (confidence 99.3%, Supplementary file 3). It also recognized several 3D structures in correspondence with the OTU 1 domain that belonged to Otubain family proteins (Supplementary file 3 shows the best recognition, confidence level: 99.8%). The region corresponding to the OTU 2 domain was not recognized. Therefore both FASTA and Phyre[2] analyses provided useful information to identify a deubiquitinating protease domain in the region upstream the OTU 1 domain (Fig. 2), which we named USP (ubiquitin-specific protease). Interestingly, the work describing a helitron in platyfish (Zhou et al., 2006)–designated HelXmac, whose structure is similar to that of Helinoto—had already noted the similarity of the N-terminal region of the first OTU-like protease domain to the tegument protein of bovine herpes virus, but it said nothing about its DUB character. To establish the similarity of the USP-like helitron domain to the viral DUB the corresponding sequences of teleost helitrons Helinoto, HelXmac, and HelDrer5 (Hel_Dr5 in Poulter et al., 2003) were compared to those of three viral USP domains (UL36, M48 and BPLF1; Schlieker et al., 2007). The alignment shown in Fig. 3 shows the aa in the viral domains that correspond to the catalytic residues and to residues conserved in the vicinity of the catalytic site. The putative USP domains in the teleost helitrons contain highly conserved residues at the positions recognized to be critical for viral DUB activity. The latter finding thus confirms the USP character of this Helinoto region. Detection of a new DUB-like domain near the two consecutive OTU domains that are homologous to the members of a different DUB family extends and strengthens the DUB character of the N-terminal portion of Helinoto-like helitron polypeptides.

The phylogenetic relationship between the Helinoto OTU and USP domains and the homologous regions of the HelDrer5 and HelXmac helitrons was explored by comparing their aa sequences to all the DUBs belonging to the cognate subfamilies found in the genome of *D. rerio* and to the Otubains of *Salmo salar*, *Oreochromis niloticus*, and *Ictalurus punctatus*, the only fish sequences retrieved from the GenBank database. We used two different approaches, ML and Bayesian analysis, which generated the phylogenetic trees reported in Supplementary file 4. They show that the Helinoto OTU and USP domains do not correspond to any known DUB subfamilies: ML and Bayesian analyses both grouped the OTU 1 and OTU 2 domains in independent clusters separate from any other OTU subclass (A and B). Notably, ML analysis grouped the Helinoto USP sequences into a cluster: these sequences were more similar to two Ub proteases (ubiquitin carboxyl-terminal hydrolases 44 and 49) than to the viral USP sequences identified by FASTA and Phyre[2] (UL36, BPLF1 and M48) (C). In contrast the Bayesian approach found a slightly closer phylogenetic relationship to the viral USP sequences (D).

### 3.3. Helinoto has transcriptional activity

#### 3.3.1. BLAST analysis of transcript databases

Characterization of the structure of Helinoto and the identification of a new functional domain made it even more interesting to establish whether the transposon is transcriptionally active. The recent submission of seven notothenioid transcriptomes (Shin et al., 2012) enabled performing a BLAST analysis for mRNAs matching the Helinoto sequence. The results of BLAST analysis against all seven notothenioid sequence read archive (SRA) libraries using the of 9882 bp H4–B9 sequence as query ("highly similar sequences", MegaBLAST program) are shown in Fig. 4. The SRA libraries yielding the largest number of hits were those from *C. aceratus* (brain and liver), which belongs to the same family as *C. hamatus*, and *P. antarcticum* (brain). The hits were distributed almost throughout the sequence, although those found in correspondence with the two OTU domains and the region between them accounted for 40% of the total. The USP and the ENDO domain regions also generated a large number of hits (8% and 8%, respectively), whereas hits for the REP and the HEL domains were few (1% and 4%, respectively). Moreover some hits (6%) involved the 5′ terminal region and many (19%) the 3′terminal region, both outside the Helinoto ORF.

Further insights were gained by comparing the whole Helinoto sequence to collections of contigs/assembled transcripts from NCBI (BLASTN 2.2.29+, "refseq_rna" and "est" databases), AntaGen (http://antagen.kopri.re.kr/blast/with.php) (Shin et al., 2012), and ChamatusBase (http://compgen.bio.unipd.it/chamatusbase/blast/) (Coppe et al., 2013). These comparisons identified several piscine mRNA sequences sharing a high similarity with Helinoto (Supplementary file 5, only the alignments of the longest transcripts are shown) and ranging from hundreds to thousands of bases in length. In one case more than 60% of the whole Helinoto sequence aligned with a long *D. rerio* transcript (ref|XM_005164917, *D. rerio* uncharacterized LOC101885531). Iteration of the analysis using portions of the Helinoto sequence confirmed these results.

#### 3.3.2. RT-PCR analysis of C. hamatus liver

RT-PCR analysis was performed first to establish whether Helinoto is transcriptionally active, beginning with *C. hamatus* liver total RNA. The regions spanned by the three primer pairs used, Help2/OTU (1259 bp), Help3/OTU (1696 bp), and REP/HEL (2412 bp), are shown in Fig. 4. The amplification patterns were compared with those from a classic PCR carried out with the same primer pairs, but using *C. hamatus* genomic DNA as template (Fig. 5). Amplicons corresponding to the Help2/OTU and Help3/OTU pairs were found in both patterns, indicating transcription of these Helinoto regions. Both amplicons correspond to the region containing the USP and the OTU 1 domains: Help3/OTU starts about 250 bp upstream the USP domain, which generated a large number of BLAST hits (Fig. 4); Help2/OTU starts in the USP domain and shares the reverse primer with Help3/OTU. The REP/HEL amplification pattern was quite complex, with amplicons ranging from a few tens bp to about 2300 bp; in contrast, the PCR using genomic DNA produced a simple pattern with two major amplicons, the longer of which was slightly smaller than that of the corresponding Helinoto region (2412 bp). Sequencing of the Help2/OTU and Help3/OTU RT-PCR amplicons confirmed the helitron origin of these transcripts (Supplementary file 6).

#### 3.3.3. Expression analysis of C. hamatus tissues

Further information about the transcriptional activity of Helinoto was gained by investigating various *C. hamatus* tissues by qPCR. The primer pairs used were designed on three Helinoto regions: the OTU 1, REP and HEL domains (see Table 1 for details). Total RNA from the brain, heart, head kidney, spleen, testis, ovary and liver was reverse-transcribed and used to determine the expression levels of the Helinoto domains compared to those of β-actin gene, which was used


**Fig. 2.** The structure of H4–B9 Helinoto. The domains identified along the sequence of H4–B9 Helinoto and the position of its continuous ORF (8826 bp) are shown.

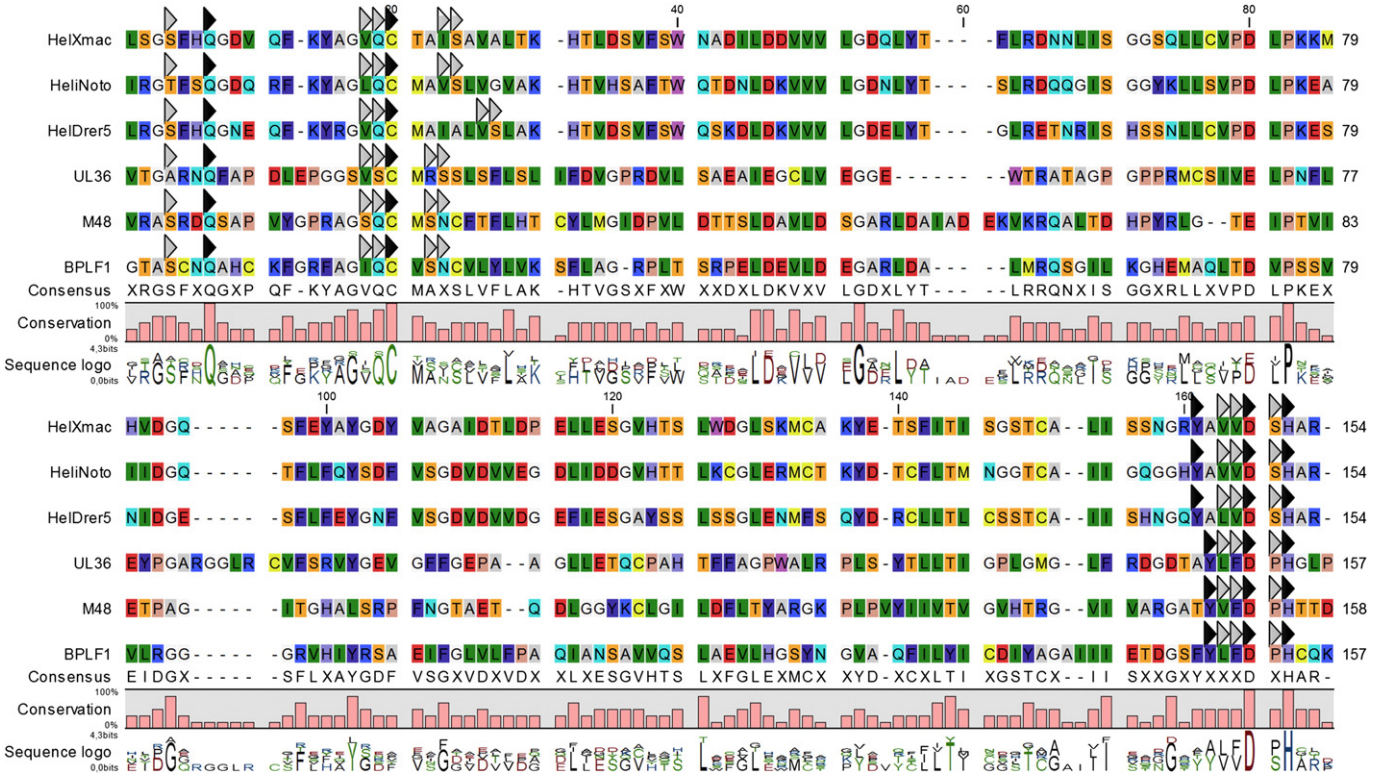


**Fig. 3.** Comparison with other USP sequences. Muscle alignment of USP domains of the helitrons Helinoto (GU014476, upgraded with the new sequences from this paper), HelXmac (Q38JW4), and HelDrer5 (BK001161) with those of the viral deubiquitinases UL36 (human herpesvirus 1, UL36 peptidase; G8H8M7), M48 (murine cytomegalovirus, tegument protein M48; H2A1S0), and BPLF1 (human herpesvirus 4–Epstein–Barr virus; YP_401652). The supposed catalytic residues (dark arrowheads) and the conserved residues in the surroundings of the catalytic site (light arrowheads) are indicated. The consensus sequence, the conservation histogram and the sequence logo are shown at the bottom of the alignment.

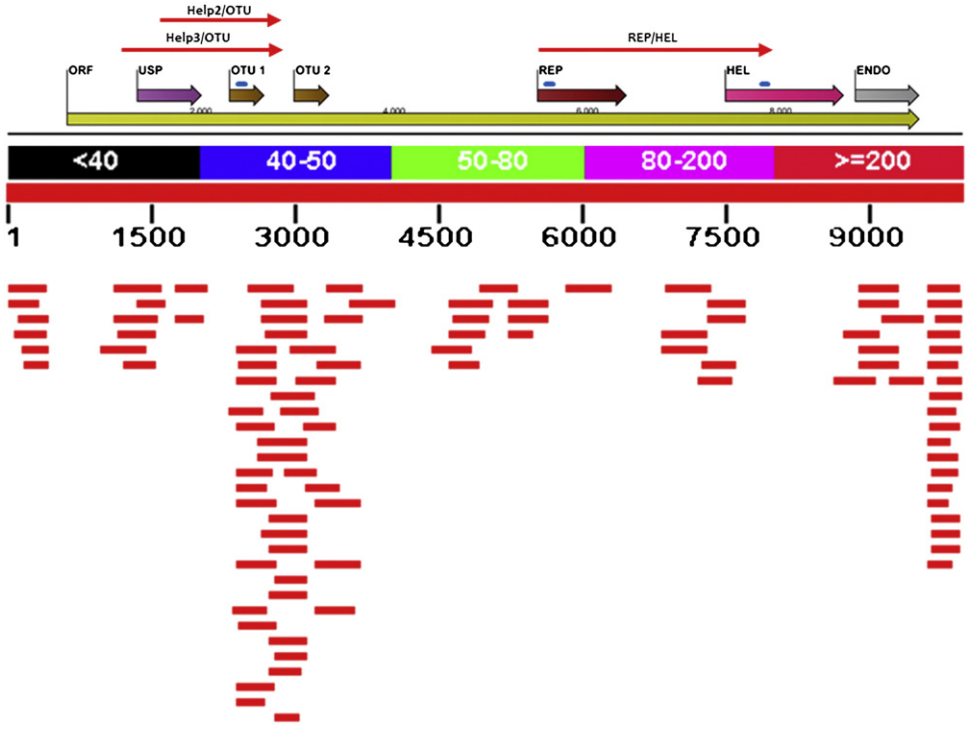


**Fig. 4.** Expression analysis. Graphic summary of the NCBI/BLAST analysis against the notothenioid SRA libraries with the distribution of 100 BLAST hits along the sequence of Helinoto. The positions of the ORF and of the helitron domains, and the regions analyzed for the transcriptional activity are shown on the top (red arrows: RT-PCR; blue segments: qPCR).

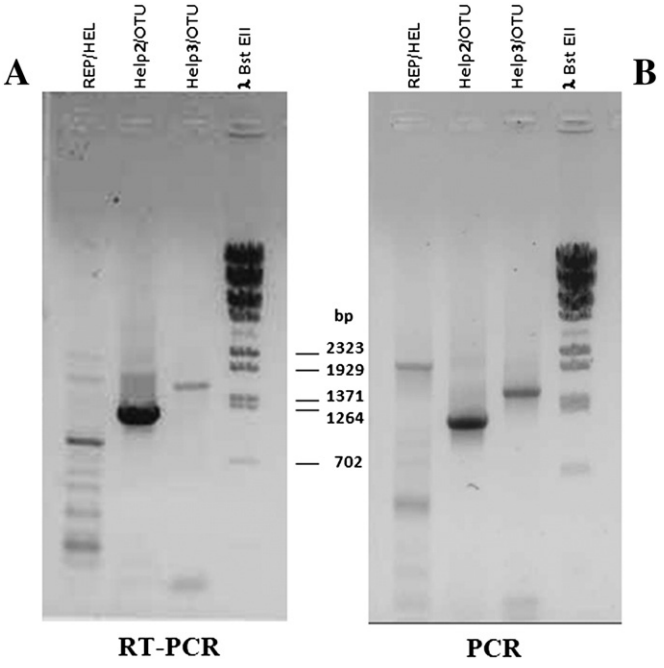


**Fig. 5.** PCR analysis. Comparison between the patterns of RT-PCR analysis on total RNA from the liver (A) and of PCR analysis on genomic DNA (B). Both were performed using the same temperature conditions and identical primer pairs (see text for details). The lanes are named with the primer pairs used in the reaction. The molecular weight marker and the size of lower fragments are shown.

for normalization. The expression patterns (Fig. 6) show that the heart and brain exhibited the highest transcriptional levels of the Helinoto regions analyzed, followed by the testis and then by all the other tissues, which contained very low amounts of Helitron transcripts. Transcripts showed different values for the three domains: they were much higher for HEL than for OTU 1 and REP in all tissues except in testis, where OTU 1 transcripts predominated.

## 4. Discussion

In a previous paper our group described for the first time a helitron, Helinoto, in the genome of the ice fish *C. hamatus* and analyzed its distribution in notothenioid families (Cocca et al., 2011). The structural markers subsequently identified at the 5′ and 3′ termini by Coates et al. (2012) now make it possible to ascribe Helinoto to the Helitron2

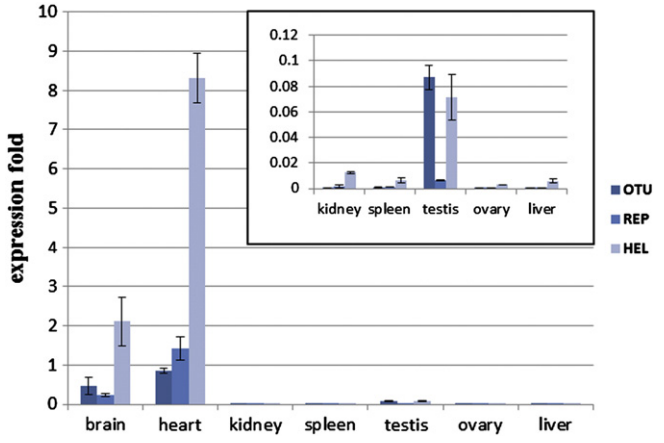


**Fig. 6.** Expression analysis of Helinoto domains. The transcriptional levels of OTU 1, REP and HEL fragments in several tissues of *C. hamatus* normalized respect to β-actin expression. All data shown are expressed as mean expression fold from triplicates. The analysis of tissues with lower expression levels is shown magnified in the inset.

group, which was first identified by Bao and Jurka (2013). These transposons are characterized by two terminal hairpins, short ATIRs at the ends, and preferential integration in proximity to gene-coding regions, as in Lep1 Helitrons. These TEs seem to play a considerable role in the creation of genetic novelty, which affects gene structure and function through the introduction of novel exon sequences (exon shuffling; Coates et al., 2012). ATIRs are short repeats measuring 8 to 15 bp; the 5′-ATIR partially coincides with the 5′-terminal hairpin sequence. The two structural motifs are probably involved in the transposition process, perhaps during the final phase of RC replication, when 5′-ATIR and 3′-ATIR pairing destroys the 5′-hairpin structure and determines the replication endpoint (Bao and Jurka, 2013). However, the conservation of hairpin structures adjacent to both Helitron2 termini suggests possible roles for these elements during transposition: in REP/HEL protein recognition or in nascent strand cleavage (Coates et al., 2012). However, further investigation is needed to clarify these aspects.

Against this background the deubiquitinating-like specific protease domain (USP) detected in the present study at the 5′ half of Helinoto upstream the two consecutive OTU-like cysteine protease domains described previously (Cocca et al., 2011) is quite interesting. The two putative OTU protease domains have been proposed to be involved in cleavage of the large helitron precursor polyprotein into functional domains (Kapitonov and Jurka, 2007); to be engaged in REP/HEL protein degradation through the ubiquitination pathway (Balakirev et al., 2003); or to activate downstream factors in the DNA repair system after or during transposition, to minimize host genome damage (Bao et al., 2010). The USP domain containing highly conserved functional residues critical for deubiquitinase activity described herein strengthens the DUB character of this portion of the helitron polypeptide.

Moreover, phylogenetic comparison with all similar *D. rerio* DUB sequences shows that the OTU and USP domains do not correspond to any of the known DUB subfamilies.

Helinoto expression analysis confirmed the presence of transcriptionally active helitrons in the genome of *C. hamatus*.

BLAST analysis of the H4-B9 construct against the SRA libraries of Notothenioidei yielded a large number of hits in correspondence with the OTU 1 and OTU 2 domains as well as with the USP and ENDO domain regions. Moreover, detection in several fish species of mRNA sequences with high similarity to Helinoto indicates that this TE family is widespread in these organisms, providing further evidence of the transcriptional activity of these genomic elements.

RT-PCR analysis was applied to investigate Helinoto expression in the DUB and the REP/HEL region. BLAST hits were very few for REP/HEL and much more numerous for the DUB region. The amplification patterns obtained from *C. hamatus* liver mRNA and genomic DNA disclosed different scenarios for the two regions even though both appear to be transcribed: whereas the DUB amplicons were consistent with the expected size, the REP/HEL signals had a complex pattern, most likely due to the effect of deletions or other mutations that have modified this portion of Helinoto. The difference between the two regions may also have influenced the output of BLAST analysis against the SRA libraries.

Quantitative expression analysis showed that the transcriptional activity of Helinoto is particularly strong in *C. hamatus* heart and brain and weaker in the testis, with different expression levels for each domain. Such uneven distribution of transcripts may depend on partial or chimeric helitron elements that can vary greatly in sequence length, and may appear after serial transposition of the elements of the same helitron family. A similar situation has been found in maize, where identification and analysis of chimeric elements derived from multiple transposition events have helped reconstruct the phylogenetic history of helitrons embedded in the maize genome (Yang and Bennetzen, 2009a).

Detection of OTU 1 transcripts in the heart, brain and testis is an interesting finding that agrees with the large number of BLAST hits in the available SRA of Notothenioidei found for the Helinoto DUB domains and with RT-PCR data.

All deubiquinating enzymes play an important role in Ub-dependent processes, and the UPS is the main pathway by which proteins are degraded in eukaryotes (Reyes-Turcu et al., 2009; Hutchins et al., 2013). In addition to Ub recycling, they are involved in processing Ub precursors, proofreading of protein ubiquitination, and disassembly of inhibitory Ub chains. The molecules with deubiquitinating activity have been classified into five families.

Genes belonging to the five established DUB families have been identified in the zebrafish genome (Tse et al., 2009). Otubains, which are involved in cellular homeostasis, have been studied in *D. rerio* (Goncharov et al., 2013). Transcriptome analysis has revealed increased levels of Ub-conjugated proteins targeted for proteasome degradation (Chen et al., 2008; Shin et al., 2012; Windisch et al., 2012) in tissues from Antarctic notothenioid and zoarcid fish compared with temperate/tropical species. These proteins are needed for protein homeostasis in the extreme Antarctic environment. Antarctic fish have unusually high levels of misfolded or damaged proteins due to their low-temperature habitat, which can affect folding rate. Since notothenioids lack a shared cell defense mechanism, such as heat shock response (Place et al., 2004), an alternative cellular protein homeostasis mechanism is required to ensure tissue functioning in Antarctic fish. Moreover, exposure to increasing cold temperature has been seen to induce up-regulation of a large group of genes involved in Ub-dependent protein catabolism and proteasome function in carp (*Cyprinus carpio*) (Gracey et al., 2004).

The new USP domain and the abundant evidence for transcriptional activity of the entire DUB region, in particular the two OTU domains, found in the present study provide new insights into this region of Helinoto. Besides suggesting a role for the USP domain in supporting the proteolytic action of the two OTU domains during transposition, it prompts another intriguing hypothesis: that acquisition of this region by Helinoto during evolution may have provided a functional advantage.

The higher OTU 1 transcriptional activity documented in the heart, brain and testis might be required to ensure correct signal transduction and normal cell function at low temperatures. Antarctic ice fish such as *C. hamatus* are the only vertebrates lacking hemoglobin or an oxygen-carrier system. The loss of hemoglobin is compensated for by a larger heart and a (fivefold) greater cardiac output compared to other fish. The mechanism of cardiac homeostasis could benefit from exonization of DUB domains to support the UPS. Cardiac homeostasis requires proper protein turnover; accordingly, impaired proteasome function could induce heart disease. An abnormal UPS has been described in human left ventricular hypertrophy and failure (Pagan et al., 2013; Rajagopalan et al., 2013). Expression of DUB enzymes has even been detected in mammalian brain and gonads. In adult mice Usp26 transcripts have been detected in different brain areas and at the same time in testis and epididymis during sexual maturation (Zhang et al., 2009). In neonatal rats UPS gene expression has been correlated with gonocyte differentiation (Manku et al., 2012). Lastly, mutations in the otu locus have been found to affect male fertility and spermatogenesis in *Drosophila melanogaster* (Tirronen et al., 1993).

In conclusion this work is part of a broader investigation into the adaptation of notothenioids to the Antarctic environment. Its findings suggest interesting research avenues. The widespread presence in the notothenioid genome of an order of DNA transposons that are still transcriptionally active in organs involved in precise physiological functions will be the subject of further investigations along with the expression of helitron domains homologous to deubiquitinase family members.

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