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Presence of an isoform of H^+-pyrophosphatase located in the alveolar sacs of a scuticociliate parasite of turbot and its physiological transcendence

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SHORT TITLE: **Two isoforms of inorganic pyrophosphatase in *Philaterides dicentrarchi***

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SUMMARY

H^+-pyrophosphatases (H^+-PPases) are integral membrane proteins that couple PPi energy with an electrochemical gradient across biological membranes and promoted the acidification of cellular compartments. In eukaryotes organisms, essentially plants and protozoan parasites, has been described the existence of various types of H^+-PPases associated to vacuoles, plasma membrane and acidic Ca^{+2} storage organelles called acidocalcisomes. In this study we achieve to draw, by staining with pH sensitive dye Lysotracker Red DND 99, the existence of two acidic cellular compartments in trophozoites of the scuticociliate marine parasite *Philasterides dicentrarchi*: the phagocytic vacuoles and the alveolar sacs. These compartments also present in its membranes H^+-PPase, which could be related with this enzyme promoting acidification of these cell structures. Furthermore, we demonstrate for the first time that the *P. dicentrarchi* H^+-PPase is constituted by two isoforms of which one, is probably generated by alternative splicing, it is localized in the membranes of the alveolar sacs showing an amino acid motif recognized by the H^+-PPase-specific antibody PAB_{HK}, and it has a high degree of conservation between aa sequences of different strains of this ciliate. Gene expression of H^+-PPase is significantly regulated by variation in salinity, indicating the role of this enzyme and the alveolar sacs in osmoregulation and salt tolerance in *P. dicentrarchi*.

Keywords: H^+-PPase, *Philasterides dicentrarchi*, alveolar sacs, osmoregulation.

KEY FINDINGS

- Philasterides dicentrarchi has at least two isoforms of H + -PPase
- The alveolar sacs are acidic structures containing an isoform of the H + -PPase
- The H + -PPase of the alveolar sacs is associated with a osmoregulatory function

INTRODUCTION

Proton-translocating inorganic pyrophosphatases (H^+-PPases) are extremely hydrophobic integral membrane proteins that utilizes the energy released upon hydrolysis of pyrophosphate (PPi), that has a high-energy phosphoandydride bound, to transport H^+ across the biological membranes against the electrochemical potential gradient (Maeshima, 2000; Belogurov and Lahti, 2002; Gaxiola *et al.* 2007; Serrano *et al.* 2004). The first discovered H^+ -PPase in membranes isolated from the photosynthetic bacterium *Rodospirillum rubrum* (Baltscheffsky *et al.* 1966), later it was located in homogenates and in higher plant vacuoles (V-H^+-PPases) as a proton pump (Karlsson, 1975), and more recently was found in acidocalcisomes of parasitic protozoa (Scott *et al.* 1998). Although for a long time it was considered that this enzyme was present only in plants and some photosynthetic bacteria (Drozdowicz *et al*, 2003.), it has now been identified in a wide range of organisms including prokaryotes extremophiles, fungi, some algae and protozoa (Maeshima 2000; Drozdowicz and Rea, 2001). In plants, V-H^+-PPases are present, in addition to the vacuole membrane (tonoplast), also in the plasma membrane (Rea and Poole, 1993; Long *et al.* 1995; Robinson *et al.* 1996). In protozoans, V-H^+-PPase is an integral membrane-associated protein that has been localized, besides to the acidocalcisomes, within the Golgi, plasma membrane, digestive vacuoles and within a microneme maturation vacuolar compartiment of apicomplexans (Harper *et al.* 2006).

The first indication of the existence of the diversity and functional heterogeneity of V-H^+-PPases was performed at the plant *Arabidopsis thaliana* observing the presence of two distinct categories del enzyme: the AVP1 and AVP2 that, after the phylogenetic analyses with other V-H^+-PPases, showing that AVP2, rather than being an isoform of AVP1, is but one representative of a novel category of AVP2-like (type II) V-PPases that coexist with AVP1-like (type I) V-H^+- PPases not only in plants, but also in apicomplexan protists such as the malarial parasites (Drozdowicz *et al.* 2000). Although there is a clear evidence for a wide occurrence of V-H^+-PPase genes in ciliates hymenostomatids, peritrichs and hypotrichs (Pérez-Castiñeira *et al.* 2002); however, until recently has only been shown the presence of V-H^+-PPase activity in the scuticociliate parasite of turbot *Philasterides dicentrarchi* (Mallo *et al.* 2015).

More specifically, in this work we report the results of a study that show for the first time the existence of a sequence variant in genes encoding two isoforms of H^+-PPase in *P. dicentrarchi.* One of these isoforms of the enzyme are predominant located in flat cortical sacs, designed “alveolar sacs” in Ciliophora, and their gene expression were modulated for the salt concentration.

MATERIALS AND METHODS

Parasites and experimental animals

Specimens of *P. dicentrarchi* (isolates B1, C1, D2, D3, I1, S1, P1; Iglesias *et al.* 2001; Budiño *et al.* 2011) were collected under aseptic conditions from ascitic fluid removed from the intraperitoneal cavity of experimentally infected turbot, *Scophthalmus maximus,* as previosly described (Paramá *et al.* 2003). The ciliates were cultured at 21ºC in complete sterile L-15 medium as previously described (Iglesias *et al.* 2003). In order to maintain the virulence of the ciliates, fish were experimentally infected every 6

months by intraperitoneal injection of 200 □L of sterile physiological saline containing 5 x10^5 trophozoites, and the ciliates were recovered from ascitic fluid and maintained in culture as described above

Turbot, of approximately 50 g body weight, were obtained from a local fish farm. The fish were kept in 250-L tanks with recirculating, aerated sea water at 14 ºC, subjected to a photoperiod of 12L:12D, and fed daily with commercial pellets (Skretting, Burgos, Spain). Fish were acclimatized to laboratory conditions for 2 weeks before the experiments were started.

Eight to 10- week-old ICR (Swiss) CD-1 mice initially supplied by Charles River Laboratories (USA) were bred and maintained in the Central Animal Facility of the University of Santiago de Compostela (Spain) following the criteria of protection, control, care and welfare of animals and the legislative requirements relating to the use of animals for experimentation (EU Directive 86/609 / EEC), the Declaration of Helsinki, and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The Institutional Animal Care and Use Committee of the University of Santiago de Compostela approved all sperimental protocols.

PCR, RT-PCR, RT-qPCR

P. dicentrarchi DNA was purified with DNAesy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. DNA was analyzed to estimate its quality, purity and concentration by A_{260} measurement in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA.)

Total RNA was isolated of *P. dicentrarchi* trophozoites with a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions after

24 hours of trophozoites incubation in culture media with different saline concentrations: 4, 8 y 37 ‰. After RNA purification, quality, purity and concentration were measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). For the cDNA synthesis (25 µL/reaction mixture), it was employed a reaction mix containing: 1.25 µM random hexamer primers (Promega), 250 µM each deoxynucleoside triphosphate (dNTP), 10mM dithiothreitol (DTT), 20U of RNase inhibitor, 2.5mM $MgCl_2$, 200U of MMLV (Moloney murine leukemia virus reverse transcriptase (Promega) in 30mM Tris and 20mM KCl (pH 8.3) and 2 µg of sample RNA. PCR (for DNA and cDNA amplification) was executed with gene-specific primers for the H^+PPase gene: forward/reverse primer pair (FPiPh/RPiPh) 5'-CGGGACCAGAGGTATCTTTTA-3' / 5'-ATTGATGTCAACGCCCCCTT-3'; and forward/ reverse primer pair (F1qPiPh/R1qPiPh) 5'-GCCTACGAAATGGTCGAAGA-3' / 5'-GCATCGGTGTATTGTCCAGA-3' for quantitative real-time reverse transcriptase PCR (RT-qPCR). In parallel, a PCR with primers for the β-tubulin gene (forward/reverse primer pair, 5'-ACCGGGGAATCTTAAACAGG-3' / 5'-GCCACCTTATCCGTCCACTA-3') was done to use β-tubulin as a reference gene (RT-qPCR). For the design and optimization of the primer sets, Primer 3Plus program was utilized, based on default parameters. PCR mixtures (25 µL) contained PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 9.0), 0.2 mM of each deoxynucleoside triphosphate (dNTPs, Roche), 0.4 mM of each primer, 3 units of recombinant Taq polymerase (NZY Taq DNA polymerase, Nzytech, Portugal) and 50 ng of genomic DNA or 2µL of cDNA. The reactions were run in automatic thermocycler (Biometra, Germany) as follows: initial desnaturing at 94ºC for 5 min; then 35 cycles at 94ºC for 30 s, 57ºC for 45 s, and 72ºC for 1 min; and finally a 7 min extension phase at 72ºC. qPCR mixtures (10 µL) contained 5-µL Maxima SYBR green

qPCR Master Mix (Thermo Scientific), the primer pair at 300 nM, 1µL of cDNA, and RNase-DNase-free water. qPCR was developed at 95°C for 5 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s ending with a melting-curve analysis at 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s. The specificity and size of PCR products were confirmed by 4% agarose gel electrophoresis. All qPCRs were performed in an Eco Real-Time PCR system (Illumina). Relative quantification of gene expression was determined by the $2^{-\Delta\Delta Ct}$ method (Livak K.J., *et al.* 2001) by using software conforming to MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines (Bustin *et al.* 2009)

Production of recombinant H^+-PPase of P. dicentrarchi in yeast cells

P. dicentrarchi RNA was purified with a NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions and cDNA synthesis was performed as indicated in the previous section. The PCR was carried out with gene-specific primers designed from a partial sequence of the H^+-PPase of *P. dicentrarchi* (Mallo *et al.* 2015) (forward/reverse primer pair 5'-AAAGAAGAAGGGGTACCTTTGGATAAAAGAattgatgtcaacgccccctt-3' / 5'-TGGGACGCTCGACGGATCAGCGGCCGCTTAGTGGTGGTGGTGGTGGTGgggac cagaggtatctttta-3'). These primers were designed and optimized by means of the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) including a hybridization region with the yeast YEpFLAG-1 (Eastman Kodak Company) plasmid and a poly His region (lower case letters correspond with the gene annealing zone). PCR reaction was developed initially at 95 °C for 5 min, and then for 30 cycles of 94 °C for 1 min, 55 °C for 1.5 min and 72 °C for 2 min. After the 30 cycles, a 7-min extension phase at 72 °C was carried out. The PCR products were purified using Gene Jet PCR

Purification Kit (Fermentas, Life Sciences) according with the manufacturer's instructions.

Purified PCR products were cloned in YEpFLAG-1 (Eastman Kodak Company) yeast expression vector, a plasmid that carries a TRP1 gene that completes the auxotrophy for the tryptophan for the host yeast (López-López *et al.* 2010).

Linearized plasmid YEpFLAG-1 by digestion with *EcoRI* and *SalI* (Takara) was used to transform *Saccharomyces cerevisiae* cells (strain BJ 3505) by the lithium acetate procedure (Ito *et al.* 1983). The procedure involves co-transformation of yeast cells with the linearized empty plasmid and the PCR-generated DNA fragment so that a recombination process occurs within the cell yielding a plasmid bearing the desired insert. Positive colonies were selected using complete medium without tryptophan (CM-Trp) containing glucose (20g/L), Yeast Nitrogen Base without amino acids medium (Sigma-Aldrich) adenine (40mg/L) and amino acids (histidine, leucine, tyrosine, 40mg/L each; arginine, methionine, threonine 10mg/L each; isoleucine and phenylalanine 60mg/L each and lysine 40mg/L).

Plasmid DNA was then extracted with Easy Yeast Plasmid Isolation Kit (Clonetech) following the manufacturer's instructions. The purified and cloned DNA fragment was subjected to sequenciation analysis (Sistemas Genómicos, Spain).

Recombinant protein of H^+-PPase of *P. dicentrarchi* was purified from transformed *Saccharomyces cerevisiae* cultures, after 72h in modified Yeast Peptone High Stability Expression Medium (YPHSM) containing 1% glucose, 3% glycerol, 1% yeast extract, and 8% peptone, at 30ºC in Erlenmeyer flasks filled with 20% volume of culture medium at 250 rpm (López-López *et al.* 2010). As inoculum, a suitable volume of a pre-culture was added to obtain an initial OD_{600} of 0.1. The cell suspension was centrifuged at 7500g for 15 min and the cleared supernatant was purified by

immobilized metal affinity chromatography on a pre-charged Ni-Sepharose Histrap column (ÄKTAprime plus, GE Healthcare Life Sciences). The column was initially equilibrated with 25 mL of binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4). After the equilibration 100mL of culture medium were charged through the column and finally, the protein bound to the column was eluted in 10 mL of elution buffer (20mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.4) (Mallo *et al.* 2015) Fractions from the elution were analyzed by 12.5% SDS-PAGE and dialyzed overnight in 2 L of bidistilled water. The dialyzed sample was concentrated in an Amicon Ultra centrifugal filter device (Millipore, USA) with a 10-kDa cut-off membrane. The final protein concentration was calculated by the Bio-Rad Protein Assay, which is based on the Bradford assay (Bradford, 1976).

Peptide synthesis

A peptide of 17 amino acids long corresponding to domain HKAAVIGDTIGDPLKDT (PAB_{HK}) of the *P. dicentrarchi* H^+-PPase were synthesized and conjugated to keyhole-limpet hemocyanin (KLH), a carrier protein, to assure maximum immunogenicity (ProteoGenix, France). A cysteine amino acid was added to two sequences to allow conjugation to KLH. The peptide were synthesized and conjugated to KLH by coupling agent sulfo-SMCC at a yield of 10-20 mg having >85% purity, lyophilized and stored at -20ºC until use.

Immunization and serum extraction

A group of five ICR (Swiss) CD-1 mice were immunized by i.p. injection with 200µL per mouse of a 1:1 (v/v) mixture of Freund complete adjuvant (Sigma-Aldrich) and a solution containing 500 µg of purified recombinant H^+-PPase and 400 µg of synthetic

peptide in PBS. The same dose of purified protein and peptide was prepared in Freund's incomplete adjuvant and injected i.p in mice 15 and 30 days after the first immunization. The mice were bled via retrobulbar venous plexus 7 days after the secondary immunization (Piazzon *et al.* 2011). The blood was left to coagulate overnight at 4º C before the serum was separated by centrifugation (2000 × g for 10 min), mixed 1:1 with glycerol and stored at −20ºC until use. In some experiments, a commercial rabbit polyclonal serum against KLH-conjugated synthetic peptide derived from *Arabidopsis thaliana* V-PPase, (anti-AVP1; UniProt P311414; Agrisera, Sweden) was also used.

Western-blot analysis

Ciliate membrane-associated proteins (MAPs) were extracted by phase separation in Triton X-114 solution (Bordier, 1981), by a previously described method (Mallo *et al.* 2013). Specifically, 10^7 cells were resuspended in 1 ml of ice-cold 10 mM Tris–HCl buffer, pH 7.5, to which 1 ml of ice-cold extraction buffer (300 mM NaCl, 20 mM Tris–HCl, pH 7.5, 2% Triton X-114) was subsequently added. The cytoskeletal elements were eliminated by centrifugation at 16000 x *g* for 10 min at 4 ºC. The supernatant was then transferred to 1.5 ml Eppendorf tubes, which were heated for 5 min at 37 ºC. At the end of this period, the solution became cloudy as a result of condensation of detergent micelles. The sample was then placed in 0.5 ml Eppendorf tubes (200 □l/tube) containing 300 □l of sucrose cushion (6% sucrose, 150 mMNaCl, 10 mMTris–HCl, pH 7.5, 0.06% Triton X-114). The detergent and aqueous phases were separated by centrifugation at 300 g for 4 min at room temperature. The resulting supernatants on the sucrose cushion of each tube were extracted carefully and mixed in new 1.5 ml Eppendorf tubes. The extraction process was repeated by adding sufficient Triton X-114

to the aqueous mixture to obtain a final concentration of 0.5%. The mixture was re-heated at 37 ºC for 5 min. Once micellar condensation had taken place, the mixture was distributed among the original Eppendorf tubes containing the sucrose cushion and the detergent phase separated in the first extraction. The tubes were then recentrifuged at 300 g for 4 min at room temperature. The resulting supernatant was discarded and the proteins contained in the detergent phase were precipitated, by adding 9 volumes of cold acetone, resuspended, by vortexing, and finally incubated for 30 min on ice. The precipitated membrane proteins were then collected by centrifugation at 16000 g for 15 min at 4 ºC and dried in a speed vacuum concentrator (MiVac, GeneVac, UK). Finally, the extracts obtained were resuspended in 10 mM Tris–HCl, pH 7.5, and stored at -80 ºC until use. The protein concentration of preparation was determined by Bradford assay.

Samples from MAPs were separated under non-reducing conditions by linear SDS-PAGE 12.5 % gels (Piazzon *et al.* 2008). After the electrophoresis, the gels were stained with Thermo Scientific GelCode Blue Safe Protein Stain (Thermo Fisher, USA) to determine qualitatively the protein bands. In parallel, a gel was submitted to immunoblotting at 15 V for 35 min to Immobilon-P transfer membranes (0.45 □m; Millipore, USA) in a trans-blot SD transfer cell (Bio-Rad, USA) with the transference buffer (48 mM Tris, 29 mM glycine, 0.037% SDS and 20% methanol, pH 9.2). The membrane was washed with Tris buffer saline (TBS; 50 mM Tris, 0.15 M NaCl, pH 7.4) and immediately stained with Ponceau S to verify transfer. After membrane distaining with bidistilled water, a blocking solution containing 0.2% Tween 20 and 3% BSA with TBS was added and the membrane was incubated for 1.5 h at room temperature. Then, it was washed in TBS and incubated overnight with anti-PAB_{HK} at 1:100 dilutions, at 4ºC. Subsequently; the membrane was washed with TBS and

incubated with rabbit anti-mouse IgG (Dakopatts; dilution 1:6000) for 1 h at room temperature. Once the membrane was washed 5 times for 5 min with TBS, it was incubated for 1 min with enhanced luminol-based chemiluminiscent substrate (Pierce ECL Western Blotting Substrate, Thermo Scientific, USA) and then visualized and photographed with a FlourChem® FC2 imaging system (Alpha Innotech, USA).

Inmmunofluorescence, Immunoelectron microscope and fluorescent stain with pH-sensitive dye

For immunolocalization of H^+-PPase isoforms, an immunofluorescence assay was performed following the protocol described previously (Mallo *et al.* 2015). Briefly, $5x10^6$ ciliates were centrifuged at 750 x *g* for 5 min, washed twice with Dulbecco's phosphate buffered saline (DPBS, Sigma Aldrich) and fixed for 5 min in a solution of 4% formaldehyde in DPBS. Following fixation, ciliates were washed twice with DPBS, resuspended in a solution containing 0.1% Triton X-100 (PBT) for 3 min and then washed twice with DPBS. Ciliates were then incubated with 1% bovine serum albumin (BSA) for 30 min. After blocking, ciliates were incubated at 4ºC overnight with a solution containing 1:100 dilutions of anti-H^+-PPase form recombinant yeast antibody and anti-PAB_{HK}. Then, ciliates were washed 3 times with DPBS followed by 1 h incubation, at room temperature; with a 1:100 dilution of FITC conjugated rabbit/goat anti-mouse/rabbit IgG-FITC antibody (Sigma). After three in DPBS, the samples were double stained with 0.8 mg/mL 4', 6-diamidine-2-phenylindole (DAPI; Sigma-Aldrich) in DPBS for 15 min at room temperature (Paramá *et al.* 2007). After three washes with DPBS samples were mounted in PBS-glycerol (1:1) and visualized by fluorescence microscopy (Zeiss Axioplan, Germany) and/or confocal microscopy (Leica TCS-SP2, LEICA Microsystems Heidelberg GmbH, Mannheim, Germany).

For immunoelectron microcopy, $5x10^6$ ciliates from cultures in exponential growth phase were centrifuged at 750 *x g* for 5 min and washed in two changes of Sörensen buffer (SB; 0.1 M sodium/potassium phosphate buffer, pH 7.3) at room temperature (RT). The resulting pellet were fixed for 60 min in 4% paraformaldehyde and 0.1% glutaraldehyde in SB at 4ºC. After the fixation, samples was washed in two changes of SB (10 min each) and incubated with 0.02 M glycine in SB for 10 min at RT. Ciliates were dehydrated in series of pre-cooled ethanol solutions (30, 50, 70, 80, 96 and 100% of 10 min each). After dehydration, the pellet were included in a mix of ethanol and resin (LR White uncatalized, Santa Cruz Biotechnology, USA; Philimonenko *et al.* 2002) 2:1 for 20 min and pure resin for 2 h. Samples were infiltrate overnight with fresh resin at 4ºC. The next day, a new exchange of fresh resin was made and allowed to polymerize at 65 ºC in vacuum for 48h. Thin sections (80 nm thich) were cut with a diamond knife on a Reichert Ultracut E (Leica Microsystems AG, Germany). Thin sections were collected on 300 mesh nickel grids (Sigma-Aldrich) and was blocked by preincubation with 10% normal goat serum (NGS) in PBS-10% albumin and 0.1% Tween-20 (PBTB) for 30 min at RT. The sections were incubated for 1 h with a primary polyclonal antibody anti-AVP1 diluted in PBTB at 1:100 dilution, washed in PBS-albumin, and incubated with 10 nm gold-labeled goat anti-rabbit IgG (Sigma) at 1:50 dilution for 60 min. Finally the sections were washed in distilled water, stained with uranyl acetate and lead citrate, and observed with a JEOL-JEM-2010 transmission electron microscope operating at 120 kV (JEOL, Japan). Controls were carried out using a non-related antibody or incubation in the presence of the secondary antibody only.

For identification of the acidic compartments on trophozoites of *P. dicentrarchi* we used a fluorescent stain assay with pH-sensitive dye Lysotracker Red DND-99.

320 $5x10^5$ ciliates were centrifuged at 700 x *g* for 5 min and washed twice with PBS
321 followed by a 10 min staining with 75 nM Lysotracker Red DND-99 (Lifetechnologies)
322 solution. After staining, ciliates were observed in a fluorescence microscopy with an
323 excitation filter BP 546 nm, dichroic mirror FT 580 nm and emission filter LP 590 nm.
324
325 *Bioinformatic and statistical analysis*
326 The aminoacid sequences obtained for the H^+-PPase gene were aligned with the
327 multiple alignments Clustal Omega program (Sievens *et al.* 2011). Genetic distances
328 were calculed to quantify sequences divergences among isolates by use of Kimura's
329 (1980) two-parameter model, as implemented in MEGA versión 6.0 (Tamura *et al.*
330 2013). Phylogenetic tree were constructed with the MEGA programme, by the
331 neighbour-joining (NJ) method applied to the Kimura two-parameter correction model
332 (Kimura 1980) by bootstraping with 1000 replicates (Felsenstein, 1985).
333 The results are expressed as means ± standard error of the mean (S.E.M.). The
334 data were examined by one-way analysis of variance (ANOVA) followed by Tukey–
335 Kramer test for multiple comparisons, and differences were considered significant at
336 $\alpha = 0.05$.
337
338 RESULTS
339 *Cellular localization of H^+-PPase of* P. dicentrarchi *in acidic compartiments*
340 Initially we cloned a cDNA fragment encoding 169 aa located between positions 305
341 and 474 of the aminoacid sequence of the H^+-PPase (GenBank accession AHH28243)
342 containing domain HKAVIGDTIGDPLKDTS in yeast expression vector YepFlag-1.
343 The mouse anti-H^+-PPase polyclonal antibodies generated following immunization with
344 the recombinant protein fragment, produces intense fluorescent staining of vacuoles

located on the back half part of trophozoites and alveolar sacs located under the plasma membrane (Fig. 1A).

Incubation of trophozoites of *P. dicentrarchi* with pH sensitive dye Lysotracker Red DND-99, produces an intense staining in the vacuoles and in the alveolar sacs (Fig. 1B).

Immunohistochemical pattern of the H^+-PPase when polyclonal antibodies PAB_{HK} were used

Indirect immunofluorescence studies using a mouse polyclonal antibody generated against a KLH-conjugated synthetic peptide of the conserved amino acid domain HKAAVIDTIGDPLKDT ($PAB_{HK;}$ Fig. 2A) and the polyclonal antibody anti-AVP, a KLH-conjugated synthetic peptide derived from *Arabidopsis thaliana* V-PPase (Fig. 3B), reveal a unique labeling on the surface of the parasite appreciate clear punctate staining pattern in the trophozoites of *P. dicentrarchi.* In immunoelectron microscopy using the polyclonal antibody anti-AVP1 clearly shows specific labeling in the membranes of the alveolar sacs (Fig. 3 C-D).

Sequence characteristics of H^+-PPase isoforms

To investigate the possible existence of various types of H^+-PPase in *P. dicentrarchi* located in the posterior vacuoles and in the alveolar sacs, we amplified a fragment of this gene by PCR and have also generated several cDNA from RNA using the pair of primers FPiPh/RPiPh. In Figure 3A, the results of nucleotide sequence amplified by PCR corresponding to an partial open reading frame (ORF) of H^+-PPase gene and its amino acid translation are shown. When analyzing on agarose gel 4% the DNA fragment amplified with primers FPiPh / RPiPh, the appearance of a single band of 558

nucleotides in size was observed; however, when a cDNA is generated from total RNA by RT-PCR and amplified with the same primers, two bands were obtained, one with an identical size to that obtained after DNA amplification (558 nucleotides) and a second band of 495 nucleotides (Fig. 3A). The sequencing of of the two bands obtained by RT-PCR showed that the nucleotide sequence of the larger fragment corresponded exactly to the sequence obtained by PCR from genomic DNA, whereas the sequencing of the minor band showed the disappearance of 63 nucleotides which is located in the largest band. After translation to aa of the two amplified fragments by RT-PCR, shows that the lower band produces a protein containing the domain complete HKAAVIGDTIGDPLKDTS, while the largest band generates a protein with this fragmented domain, containing an internal sequence of 21 amino acids (Fig. 3A).

Polyclonal antibodies generated in mice after immunization with the synthetic peptide corresponding to domain PAB_{HK}, recognized on MAPs in Western blot a single protein band of approximately 60 kD (Fig. 3B).

Phylogenetic analysis of H^+-PPases in several strains of P. dicentrarchi

To determine the degree of phylogenetic evolution between isolates of *P. dicentrarchi*, we amplified by PCR the DNA of seven isolates using the primers pair FPiPh / RPiPh. After obtaining the nucleotide sequence of each isolate and its translation into aa, was carried out a multiple alignment of the amino acid sequences using the Clustal Omega program, showing a very high degree of conservation between aa sequences of the isolates analyzed (Fig. 4A). This high level of conservation in aa sequences results in the existence of a low genetic distance between isolates and, when the phylogenetic tree using the NJ method is constructed, it is noted that five isolates have 100% homology (I1, B1, D3, P1 and S1 isolates), whereas D2 and C1 constitute two phylogenetically different groups (Fig. 4B).

Effect of salt concentration on the expression of H^+-PPase

The assays on the expression levels of RNA corresponding to the H^+-PPase of *P. dicentrarchi* trophozoites cultivated in a saline medium containing different concentrations of NaCl: between 4, 8 and 37 ‰, are shown in Fig.5. Relative mRNA levels, quantified by qPCR, of H^+-PPase remain unchanged at NaCl concentrations between 8 and 37 ‰; however when the medium contains low concentrations of NaCl (such as 4‰), a significant increase in the expression of H^+-PPase, relative to levels obtained in ciliates then incubated at concentrations of NaCl between 8 and 37 ‰ (Fig. 5).

DISCUSSION

H^+-PPases are enzymes that translocates H^+ across a membrane by using potential energy liberated on hydrolysis of the phosphoanhydride bond of inorganic phosphate (Read and Poole, 1993). They are widely distributed among land plants and have been found in several of protozoan parasites including the scuticociliate parasite of turbot, *P. dicentrarchi* (Mallo *et al.* 2015). In eukaryotes, H^+-PPases are associated to certain acidic compartments of the endomembrane system, namely, the vacuole and lysosomes of plant cells and the acidocalcisomes of trypanosomatides and apicomplexans protozoan (Pérez-Castiñeira *et al.* 2002; Scott and Docampo, 2000; Docampo *et al.* 2005). Some functions of the acidocalcisomes are the storage of cations, Ca^{2+} homeostasis, mainteinance of intracellular pH homeostasis and osmoregulation (Moreno and Docampo, 2009). In parasitic protozoans, acidocalcisomes also interact with other organelles as the contractile vacuole and other vacuoles associated with the endosomal/lysosomal pathway (Moreno and Docampo, 2009; Docampo *et al.* 2010). In

ciliates, such as *Paramecium caudatum,* has been described acidification of phagocytic vacuoles occurs through fusion nonlysosomal vesicles, named acidosomes, with the newly released vacules and these vesicles accumulate neutral red as well as acridine orange, two observations that demonstrate their acid content (Allen and Fok, 1983). Sequencing of the whole genome of several species of ciliates enable the identification of genes encoding the V-ATPase, a proton pump that drives H^+ across membranes, and that is crucial as an acidifier of food vacuoles (Plattner, 2010); however, although there is evidence of the existence of H^+-PPases in ciliates, since there are several sequences deposited in nucleotide databases (eg. *Tetrahymena thermophila,* GenBank accession XM_001011583; *Tetrahymena pyriformis,* GenBank accession AJ251772), it available thus far too little information on the occurrence of membrane-bound H^+-PPases and their physiological role in these Protozoa (Pérez-Castiñeira *et al.* 2001). Although acidocalcisomes as a whole and some of their transport activities have not been characterized in ciliates as yet, where they may also occur (Plattner *et al.* 2012). In this study it is clearly evident that the H^+-PPase in *P. dicentrarchi* colocalises both phagocytic vacuoles and in the alveolar sacs, and these two structures are acidic cellular components which are stained with the pH sensitive dye Lysotracker Red DND 99.

Altohough subcellular localization of members of the H^+-PPase family is mainly in endocellular membranes (vacuolar tonoplast) and acidocalcisomal membranes of eukaryotes (algae, plants and protozoa) (Maeshima, 2000; Drozdowicz and Rea, 2001; Docampo *et al.* 2005) and plasma membrane invaginations of both bacteria and archaea (Baltscheffsky *et al.* 1999; Serrano *et al.* 2004), evidence for a differential subcellular localization of the AVP1 (vacuole) and AVP2 (Golgi complex and lysosomes) isoforms has been only reported in plant cells (Rea *et al.* 1992; Mitsuda *et al.* 2001). Indirect immunofluorescence microscope with polyclonal antibodies to investigate the

446 subcellular localization of V-H^+-PPase in *P. falciparum* indicated that VP1 is present
447 within the vacuolar membrane and, possibly, in food vacuoles (Luo *et al.* 1999;
448 MacIntosh *et al.* 2001) and it seems that the proton pumps V-H^+-PPase and V-H^+-
449 ATPase are colocalized in acidic organelles in malarian parasites including
450 acidocalciomes and food vacuoles (Marchesini *et al.* 2000; Saliba *et al.* 2003;
451 Moriyama *et al.* 2003). In our study, we demonstrate by immunofluorescence and
452 immunohistochemistry to TEM that the PAB_{HK} sera, which recognizes the highly
453 conserved domain HKAAVIDTIGDPKDT, It generates a specific labeling only on the
454 alveolar sacs of ciliates, which suggests that this domain is not found, or not recognized
455 in the H^+-PPase vacuoles. Thus, immunostaining with PAB_{HK} could be evidencing the
456 possible existence of two isoforms of H + -PPase in *P. dicentrarchi*.

457 The existence of multiple H^+-PPases isoforms is clearly demonstrated in plants
458 (Venter *et al.* 2006). Thus for example, in rice (*Oryza sativa* L.) genome have been
459 detected at least two genes encoding the H^+-PPase (Sakakibara *et al.* 1995), three
460 isoforms in tobacco (Lerchl *et al.* 1995), two isoforms in red beet (*Beta vulgaris* L.)
461 (Kim *et al.* 1994), two isoforms in barley (*Hordeum vulgare* L.) (Fukuda *et al.* 2004),
462 two isoforms in grapevine (*Vitis vinífera* L.) (Venter *et al.* 2006), two isoforms in cacao
463 (*Theobroma cacao* L.) (Motamayor *et al.* 2013), with highly homologous within the
464 coding región but differs strongly in the unstranslated regions and their expression are
465 probably regulated in a different manner (Maeshima, 2000). Plants have two
466 phylogenetically distinct V-H^+-PPases that can be classified into two subclases, AVP1,
467 that depend on cytosolic K^+ for their activity and are moderately sensitive to inhibition
468 by Ca^{2+} and AVP2, wich are K^+-independent but extremely Ca^{+2}-sensitive (Sarafian *et*
469 *al.* 1992; Drozdowicz *et al.* 2000; Gaxiola *et al.* 2007). Parasites, such as in the
470 malarian parasite *Plasmodium falciparum*, also two genes encoding corresponding VP1

and VP2 have been identified (MacIntosh *et al.* 2001), and in ciliates also available in the databases of the sequence corresponding to an isoform 2 of himenostomatid *Tetrahymena pyriformis* (GenBank accession AJ251471).

There is a near-complete conservation between AVP1/AVP2 of the aminoacids sequences recognized by polyclonal antibody PAB_{HK} (HKAAVIGDTIGPLK) that provides further justification for the proposal that these antibodies are universal reagents for the detection of V-PPase polypeptides (Drozdowicz and Rea, 2001). We have previously shown that H^+ -PPase of *P. dicentrarchi* showed a common motif with the polyclonal antibody PAB_{HK} specific to AVP1 (Mallo *et al.* 2015). Specifically, in this study we found that this motif is encoded by a gene containing an intercalated nucleotide sequence to be transcribed into RNA generates two isoforms: one of which produces a protein with the fragmented motif and other isoform produces a protein containing the complete motif. The total sizes of the proteins produced by gene isoforms would vary between 62-64 kD (2kD difference is the estimated size of the peptide intercalated between the motif PAB_{HK}), but an analysis by SDS-PAGE or Western blot probably would go unnoticed due to the limitation of this technique for separating proteins of molecular sizes very close (Dauly *et al.* 2006). The hypothesis proposed in this paper to explain the presence of the two H^+-PPase isoforms is based, on the one hand in the differential recognition of the H^+-PPase in the alveolar sacs by PAB_{HK} antibodies, and secondly, in the presence of two amino acid sequences in the cDNAs generated by RT-PCR with primers FPiPh / RPiPh. The presence of the two isoform containing the complete PAB_{HK} motif in the H^+-PPase in the alveolar sacs could be explained by the existence of an alternative splicing, while isoform 1 of H^+-PPase present in the vacuoles not suffer this process and generate a protein with fragmented motif it would not be recognized by the polyclonal anti-

HKAAVIGDTIGPLKDT (PAB_{HK}). There are some examples of genes that generate isoforms transcribed from alternate promoter sites within the gen which may mediate cell signaling and induce their translocation to various cellular localizations (Saito *et al.* 2002). Furthermore, it is also well known that the splicing regulation can be modulated by several sequence elements in both exons and introns that either actívate (exonic splicing enhancer, ESE; introning splicing enhancer, ISE), or repress (exonic splicing silencer, ESS; intronic splicing silencer, ISS) (Poulos *et al.* 2011).

The description of nucleotide sequences of H^+-PPase genes from plants, bacteria and archaea brought forward an unusually high degree of sequence conservation (Serrano *et al.* 2007). In our study, we also found a high level of squence conservation of the H^+-PPase gene among several isolates of *P. dicentrarchi* which also could also be used, conjunction with other highly conserved genes such as the □-tubulin, for detecting intraspecific genetic variation within populations of scuticociliates that infect cultured turbot (Budiño *et al.* 2011).

In plants, it is well established that the efficient exclussion of Na^+ excess from the cytoplasm and vacuolar Na^+ accumulation are the most important steps towards the maintenance of ion homeostasis inside the cell, and both tonoplast and plasma membrane Na^+/H^+ antiporters exclude Na^+ from the cytosol driven by the H^+-motive force generated by the plasma membrane H^+-ATPase and H^+-PPase (Silva and Gerós, 2009). Algal and plant H^+-PPases are induced under anoxia, chilling and salt stresses (Carystinos *et al.* 1995; Fukuda *et al.* 2004), and overexpression of the vacuolar H^+-PPase isoform AVP1 in the model plant *Arabidopsis* has been claimed to confer increased saline and drought tolerance (Gaxiola *et al.* 2001). Ciliates are eurihalins organisms particullarly well adapt to salinity changes, can live in salinities as low as 4‰ and as high as 62‰ (27‰ higher than seawater) (Hu, 2014). In our study, we found

that *P. dicentrarchi* is able to respond to salinity stress with changes in the expression of H^+-PPase located in the alveolar sacs indicating a potential role of these structures in salt tolerance by marine scuticociliates.

In conclusión, the H^+-PPase of *P. dicentrarchi* is located in the membranes of the phagocytic vacuole and alveolar sacs promoting the acidification of these cellular compartments. Specifically, in the alveolar sacs are located a isoform of ionic pump H^+-PPase containing a highly conserved aa motif generated by alternative splicing process, that is recognized by polyclonal antibodies PAB_{HK}, and whose gene expression is regulated under conditions altered salt, which suggest that these structures must play an important physiological role in the adaptative responses of these marine ciliates to mainteinance of both intracellular pH homeostasis and osmoregulation.

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FIGURE LEGENDS

Figure 1. A, B) Inmunofluorescence detection by confocal microscopy of H^+-PPase in a ciliate trophozoite using a mouse antibody anti- recombinant H^+-PPase expressed in yeast *Saccharomyces cerevisiae* and a secondary polyclonal antibody anti-mouse Ig conjugated with FITC. After immunofluorescence assay, trophozoites were counterstained with DAPI to identify the macronucleus (M). A) Arrows indicate the immunolocalization of H^+-PPase in the vacuoles and arrowhead indicate the presence of a specific immunostaining appears as a dotted line coinciding with the alveolar sacs. B) Fluorescent staining of acidic compartiements of *P. dicentrarchi* using Lysotracker Red DND-99 show a fluorescent staining of the posterior vacuoles (arrows) and the alveolar sacs (arrowhead). Scale bars = 10 μm.

Figure 2. Inmunofluorescence detection by confocal microscopy of H^+-PPase isoform (H^+-PPase2) with PAB_{HK} polyclonal antibody (A) and anti-AVP1 polyclonal antisera (B) where you can see a pattern of discontinuous fluorescence on the surface of trophozoites (arrowhead). C,D) Inmmunoelectromicroscopy localization of the isoform H^+-PPase2 using the polyclonal PAB_{HK} antibody corresponding to AVP1 isoform of *A. thaliana* and which shows a specific staining (arrowhead) on the membrane of the alveolar sacs.

Figure 3. A) Nucleotide sequence belonging to a gene region of the H^+-PPase of *P. dicentrarchi* together with its corresponding translation into amino acids, containing a sequence motif recognized by the antibody PAB_{HK} (box in black). In the lower part of the figure are shown the products of PCR and RT-PCR obtained usign ADN and RNA

767 as template and the primers FPiPh/RPiPh, analyzed on agarose gel 4% being observed
768 in the case of using cDNA as template, the presence of two bands that correspond,
769 respectively to isoform 1 (the larger), and 2 (the smallest) and containing this last the
770 complete motif recognized by the polyclonal antibody PAB_{HK}. B) Western blot with
771 antibody PAB_{HK} (lane 1) on ciliate membrane-associated proteins (MAPs) of
772 trophozoites subjected to SDS-PAGE under nonreducing conditions and which
773 recognizes a single band of approximately 62 kD (arrow). Mw: Molecular weight
774 markers.
775
776 **Figure 4.** A) Multiple sequence alignment using Clustal Omega program of aa
777 sequences obtained from a partial ORF of the gene of H^+-PPasa from several isolates
778 (B_1, D_2, D_3, C_1, I_1, S_1 y P_1) of *P. dicentrarchi.* The boxes in bold indicate the motif
779 recognized by the antibody PAB_{HK}. B) Phylogenetic comparison of H^+-PPase of *P.*
780 *dicentrarchi* isolates. Aligned Aa sequences were sujected to phylogenic analysis with
781 neighbor joining (NJ) method. The numbers at the nodes represent boostrap values out
782 of 1000 resampled values in the NJ analysis with the Kimura two-parameter correction
783 model.
784
785 **Figure 5.** Relative gene expression levels of H^+-PPase of *P. dicentrarchi* determined by
786 RT-qPCR in trophozoites incubated for 24 h in medium containing different
787 concentrations of NaCl: 4, 8 and 37 ‰. Gene expression was normalized to reference
788 gene □-tubulin of *P. dicentrarchi* and normalized data are expressed in arbitrary units.
789 Values shown are means ± standard error (E.S.) of five assays. *$P < 0.01$ relative to
790 ciliates incubed in the medium containing a salt concentration of 37 ‰.

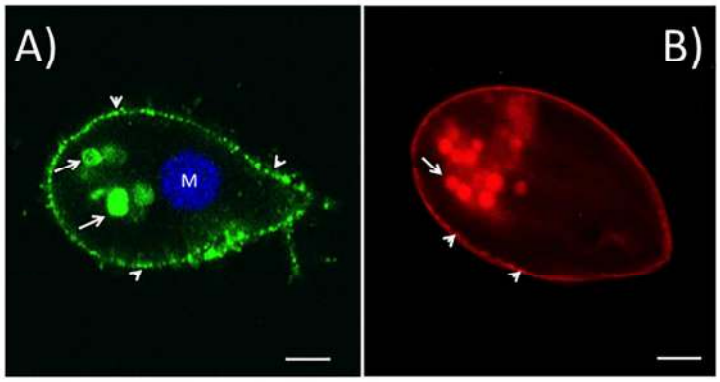

Figure 1

119x90mm (300 x 300 DPI)

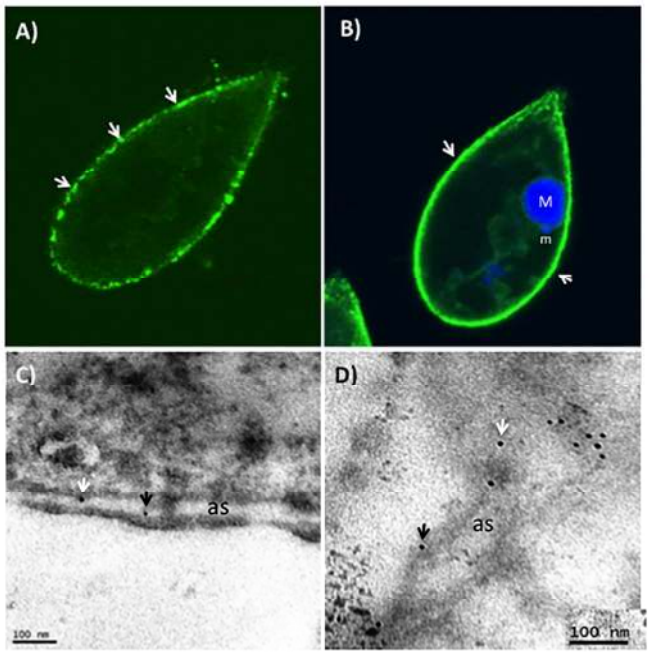

Figure 2

119x90mm (300 x 300 DPI)

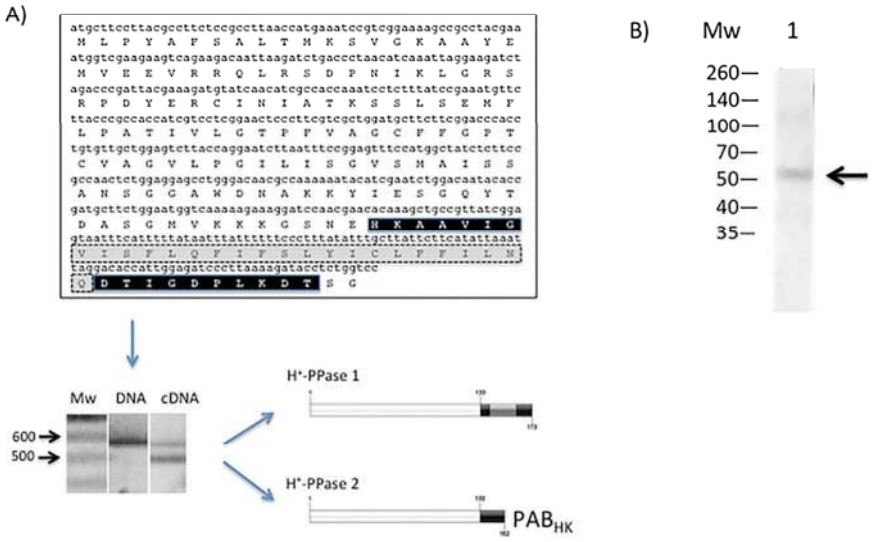

Figure 3

90x67mm (300 x 300 DPI)

A)

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D2      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
I1      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
B1      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
D3      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
P1      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
S1      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
C1      MLPYAFSALTMKSVGKAAYEMVEEVRRQLRSDPNIKLGRSRPDYERCINIATKSSLSEMF
        ************************************************************

D2      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
I1      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
B1      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
D3      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
P1      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
S1      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
C1      LPATIVLGTPFVAGCFFGPTCVAGVLPGILISGVSMAISSANSGGAWDNAKKYIESGQYT
        ************************************************************

D2      DASGMVKKKGSNEHKAAVIGVISFFKFIFSLYICLFFILNQDTIGDPLKDTSG
I1      DASGMVKKKGSNEHKAAVIGVISFLQFIFSLYICLFFILNQDTIGDPLKDTSG
B1      DASGMVKKKGSNEHKAAVIGVISFLQFIFSLYICLFFILNQDTIGDPLKDTSG
D3      DASGMVKKKGSNEHKAAVIGVISFLQFIFSLYICLFFILNQDTIGDPLKDTSG
P1      DASGMVKKKGSNEHKAAVIGVISFLQFIFSLYICLFFILNQDTIGDPLKDTSG
S1      DASGMVKKKGSNEHKAAVIGVISFLQFIFSLYICLFFILNQDTIGDPLKDTSG
C1      DASGMVKKKGSNEHKAAVIGVISFLKFIFSLYICLFFILNQDTIGDPLKDTSG
        ************************:***************************
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B)

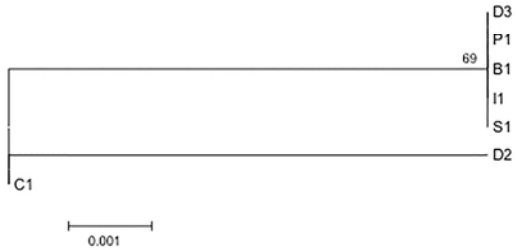

Figure 4

90x67mm (300 x 300 DPI)

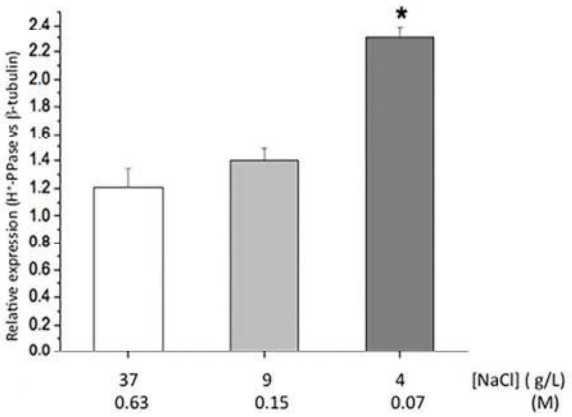

Figure 5

90x67mm (300 x 300 DPI)