

Cloning, Characterization, and Expression Levels of the *Nectin* Gene from the Tube Feet of the Sea Urchin *Paracentrotus Lividus*

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Abstract Marine bioadhesives perform in ways that manmade products simply cannot match, especially in wet environments. Despite their technological potential, bioadhesive molecular mechanisms are still largely understudied, and sea urchin adhesion is no exception. These animals inhabit wave-swept shores, relying on specialized adhesive organs, tube feet, composed by an adhesive disc and a motile stem. The disc encloses a duo-gland adhesive system, producing adhesive and deadhesive secretions for strong reversible substratum attachment. The disclosure of sea urchin *Paracentrotus lividus* tube foot disc proteome led to the identification of a secreted adhesion protein, *Nectin*, never before reported in adult adhesive organs but, that given its adhesive function in eggs/embryos, was pointed out as a

putative substratum adhesive protein in adults. To further understand *Nectin* involvement in sea urchin adhesion, *Nectin* cDNA was amplified for the first time from *P. lividus* adhesive organs, showing that not only the known *Nectin* mRNA, called *Nectin-1* (GenBank AJ578435), is expressed in the adults tube feet but also a new mRNA sequence, called *Nectin-2* (GenBank KT351732), differing in 15 missense nucleotide substitutions. *Nectin* genomic DNA was also obtained for the first time, indicating that both *Nectin-1* and *Nectin-2* derive from a single gene. In addition, expression analysis showed that both *Nectins* are overexpressed in tube feet discs, its expression being significantly higher in tube feet discs from sea urchins just after collection from the field relative to sea urchin from aquarium. These data further advocate for *Nectin* involvement in sea urchin reversible adhesion, suggesting that its expression might be regulated according to the hydrodynamic conditions.

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Introduction

Currently, there is a significant interest in marine adhesion and adhesives because the bonding properties of marine bioadhesives are still unmatched by commercial adhesive systems, especially in wet environments, making them suitable for numerous industrial and medical applications (Del Campo et al. 2013). Despite this interest, adhesive mechanisms are still understudied, especially in temporarily attached marine organisms, such as sea urchins. These animals were shown to attach with tenacities (attachment force per unit area) similar to other marine and commercial adhesives, being effective on several substrata with variable chemistry and roughness (Santos et al. 2005; Santos and

Flammang 2006, 2008), thus reinforcing the biotechnological potential of their adhesives.

Sea urchins, as other echinoderms, possess specialized adhesive organs—adoral tube feet, composed of an enlarged and flattened apical disc, which is connected to the animal body by an extensible tether, the stem. Together, they form a functional unit, the stem allowing the tube foot to lengthen, flex, and retract, whereas the disc makes contact with and adheres to the substratum (Santos and Flammang 2006). The disc encloses a duo-gland adhesive system, producing separately adhesive and deadhesive secretions. Thus, it can attach and detach repeatedly, leaving an adhesive footprint in the substratum after detachment.

Nowadays, little is still known on the molecular mechanisms behind sea urchin reversible adhesion and recent studies have focused on the analysis of either the footprint constituents or the proteome of the adhesive organs. *Paracentrotus lividus* adhesive secretion was shown to be made up, in dry weight, of proteins (6.4 %), lipids (2.5 %), carbohydrates (1.2 %), and a large inorganic fraction (45.5 %). However, in terms of adhesive proteins, all it is known is that the adhesive secretion contains actins, tubulins, and histones, most likely contaminants from disc epidermal cells (Santos et al. 2009). These same proteins were identified in the sea star footprint proteome as well, but so far only one protein, Sea star footprint protein 1 (Sfp1), was unequivocally assigned as a constituent of the sea star adhesive secretion (Hennebert et al. 2014). As for the deadhesive secretion, it is believed to contain enzymes that would explain the presence of an adhesive footprint in the substratum each time the disc detaches. Indeed, in the sea star adhesive footprint proteome, two enzymes with metalloendopeptidase activity were identified that could be involved in the detachment process by degradation of the adhesive proteins (Hennebert et al. 2015).

The first characterization of the proteome of *P. lividus* tube foot discs, which contains the precursors of the adhesive and deadhesive proteins, provided new information, but since tube foot discs present a complex histological structure (Santos and Flammang 2006), the identified putative adhesive proteins represented only 2 % of the total proteome (Santos et al. 2013). Within these was a secreted cell/substratum adhesion protein, *Nectin* (GenBank AJ578435), previously identified in eggs/embryos of the same species but never before reported in adult adhesive organs (Santos et al. 2013). This protein was reported to be secreted during embryogenesis becoming part of the embryonic extracellular matrix and to significantly increase the binding of embryonic dissociated cells to the substratum when coated on polystyrene substratum (Matranga et al. 1992). Therefore, given its function in eggs/embryos, *Nectin* was pointed out as a putative substratum adhesive protein in adults (Santos et al. 2013). In addition, it was hypothesized that *Nectin* could simultaneously ensure the cohesion of the adhesive components, binding proteins and

carbohydrates (Santos et al. 2009), since it possesses six galactose-binding discoidin-like (DS) domains that can bind molecules bearing galactose and N-acetylglucosamine carbohydrate moieties (Costa et al. 2010).

Although highly homologous to egg/embryo *Nectin*, the newly identified adult tube feet disc *Nectin* presented sequence differences, namely one isoform with the predicted molecular mass (~108 kDa; under reducing conditions) but a more acidic isoelectric point (~4.0 rather than ~6.0), and several isoforms with variable degrees of posttranslational modifications (phosphorylation and glycosylation) (Santos et al. 2013). These sequence differences could be explained either by gene duplication or alternative splicing since both mechanisms of proteome diversity are conserved in echinoderms (Xiang et al. 1988; Mistry et al. 2003).

To further investigate *Nectin* involvement in sea urchin reversible adhesion, we hereby present the first description of the amplification from adult *P. lividus* sea urchin tube feet of the cDNA encoding egg/embryo (GenBank AJ578435) but also a new *Nectin* sequence (GenBank KT351732), named for simplicity *Nectin-1* and *Nectin-2*, respectively. In addition, we report the analysis of *Nectin* genomic DNA, mRNA quantitative expression, and transcript tissue localization, providing further evidences supporting *Nectin* adhesive role in sea urchin reversible attachment.

Materials and Methods

Sea Urchins and Samples Collection

Sea urchins from the species *P. lividus* (Lamarck, 1816) were collected at low tide on the west coast of Portugal (Estoril, Cascais). After collection, the animals were transported to Vasco da Gama Aquarium (Dafundo, Oeiras) and kept in open-circuit tanks at 15 °C and 33 ‰ until tube feet collection. To collect the adhesive tube feet discs and stems, sea urchins were placed in smaller plastic aquariums (3 L) filled with seawater and with aeration. These aquaria were covered internally with removable glass plates to which animals were allowed to attach and then forced to detach, causing a number of tube feet to break by the stem. Broken tube feet were collected (either attached to the glass plates or floating in the aquarium water), pooled and stored in TE buffer (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA; Invitrogen) at –80 °C until further use. This procedure allowed minimizing individual variability since RNA isolation was obtained from pools of hundreds of tube feet belonging to 40 individuals. Some of these tube feet were dissected to separate discs from stems (working in Petri dishes placed on ice) and then stored as above. For expression analysis purposes, tube feet were also obtained just after sea urchin collection from the field and then stored as above described. For genomic DNA isolation,

gonads from five individuals were also collected and stored using the same procedure.

Total RNA Isolation and *Nectin* cDNA Synthesis

Tissue samples were defrosted on ice, suspended in Trizol (Invitrogen), and homogenized at 15,000 rpm with a shaft-type homogenizer (Ystral). Total RNA was isolated according to the manufacturer's instructions of Trizol reagent and purified with Purelink RNA Micro Kit (Invitrogen). The mRNA was isolated from total RNA using Oligotex mRNA Kit (Quiagen) and used for the first-strand cDNA synthesis. cDNA was obtained using random hexamer primers and Superscript III reverse transcriptase with the SuperScript™ First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. The obtained cDNA was used as template for amplification of *Nectin* coding gene.

Nectin full-length coding gene

Full-length *Nectin* coding gene was amplified from adult *P. lividus* sea urchin tube feet, using flanking primers (*fwr*-ATGGCGATATCACATAATGCGTTAATGG and *rev*-CTCACTCTGTGAGAGGACCTTCC) designed based on the sequence of egg/embryo *Nectin* (*Nectin-1*; GenBank AJ578435). The following conditions were used: 35 cycles at 94 °C for 5 s, 55 °C for 30 s, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. PCR products were inserted into the pCR-XL-TOPO vector using TOPO® XL PCR Cloning Kit (Invitrogen), transformed in chemically competent TOP10 *Escherichia coli* cells according to the manufacturer's instructions, and five positive clones were selected to confirm *Nectin* nucleotide sequence. For the latter, three internal fragments with predicted amplicon sizes of 771, 1338, and 998 bp (1 to 771 bp—*nec1*; 710 to 2048 bp—*nec2*; and 1968 to 2956 bp—*nec3*) were obtained. The primer sequences were as follows: *Nec1* *fwr*-ATGGCGATATCACATAATGCGTTAATGG and *rev*-CTGGCGACCCTGCGTAAATAACTC; *Nec2* *fwr*-GCTGCAAGGTGACCTTAACAGC and *ver*-GTGGAAGCCGTATATGCACTGTC; *nec3* *fwr*-CCGACGACATTGGCGGGTTC and *rev*-CTCACTCTGTGAGAGGACCTTCC.

Nectin PCR-RFLP

Predictions and simulations of the restriction fragment length of *Nectin* reference sequence (GenBank AJ578435) was performed by online restriction mapping using RestrictionMapper software (<http://www.restrictionmapper.org/>) with around 200 different endonuclease recognition sites. Enzymes that potentially produce informative fragments were selected after inspection of the predicted

restriction positions and calculation of the produced fragment lengths between *Nectin-1* (GenBank AJ578435) and *Nectin-2* (GenBank KT351732) amplified from urchin tube feet. For PCR-restriction fragment length polymorphism (RFLP) assay, cDNA obtained from tube feet of a single individual was used. The FastDigest *SsiI* restriction enzyme (Thermo Scientific) was selected based on *in silico* analyses and used in reactions in a final volume of 20 µl containing the 1× buffer recommended by the manufacturer, 3 U of the enzyme, and 200 ng of PCR product, incubated at 37 °C for 4 h. Digested cDNAs were size-fractionated on a 1.2 % agarose gel, fragment patterns were revealed in ethidium bromide solution and gel was visualized by Gel Doc XR imaging system (Bio-Rad). In total, amplified fragments of 20 individuals were analyzed.

Bioinformatic Analysis

Protein motifs were identified using SMART (<http://smart.embl-heidelberg.de/>) and the conserved domain database from NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The signal peptide was predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), and the theoretical isoelectric point and molecular weight were predicted using Compute pI/MW (<http://expasy.org/tools/protparam.html>). Disulfide bonds were identified with ScanProsite (<http://expasy.org/tools/scanprosite/>), and hydrophobicity profile analysis was performed using the Kyte-Doolittle method (<http://www.expasy.org/cgi-bin/protscale.p1>). Sequence similarity was analyzed using BLAST from NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multisequence analysis of putative transcription variations was performed using CLUSTALW in BioEdit 7.0. Putative phosphorylation of serine, threonine, or tyrosine residues was predicted using the Phosphorylation Sites Predictor DISPHOS 1.3 (<http://www.dabi.temple.edu/disphos/>), and the putative glycosylation sites were predicted using the *in silico* platform for prediction of N-, O- and C-glycosites in eukaryotic protein sequences (GlycoEP, <http://www.imtech.res.in/raghava/glycoep/submit.html>).

Identification of *Nectin* Gene

Genomic DNA was obtained from *P. lividus* gonads. Tissue was homogenized with a shaft type homogenizer (Ystral) on ice in 50 mM Tris-HCl with 0.4 mM NaCl and 0.1 M EDTA, pH 8, and final addition of 0.5 % SDS. Equal volume of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed, and centrifuged at 12,000 g for 5 min. The aqueous phase was transferred to another tube and an equal volume of 100 % chloroform was added followed by centrifugation at 12,000 g for 5 min. DNA in the supernatant was precipitated using 2.5 volumes of ethanol and centrifuged at

12,000 g for 10 min. Pellet was washed with 70 % ethanol, dried, and then resuspended in water. DNA was treated with 10 mg/ml of RNase for 30 min at 37 °C and its integrity checked in a 0.8 % agarose gel. In order to identify the *Nectin* gene, the fragments *nec-1*, *nec-2*, and *nec-3* were amplified from genomic DNA using the above-described primers. Just the fragments *nec-2* and *nec-3* presented amplicon sizes higher than predicted and therefore were cloned in pCR-XL-TOPO vector and transformed in competent TOP10 *E. coli* cells. The positive clones were sequenced using a primer walking sequencing method. Briefly, the primers that bound to the 3' or 5' extremity of the insert (*Nec2 fwr* and *Nec2rev* in *nec-2* or *Nec3fwr* and *Nec3rev* in *nec-3*) were used to synthesize a short DNA strand adjacent to the unknown sequence. Then, the new short DNA strand was sequenced using the chain termination method and the end of the sequenced strand was used as a primer for the next part of the long DNA sequence. A total of six pairs of primers were used to cover all gene sequence (Supplementary Table 1).

Probe Design and Synthesis

The RNAfold web server was used to predict secondary structures in dot-bracket notation, in order to select the most convenient template region of *Nectin* to synthesize a probe with a minimum free energy. Probes were generated from a 472-bp fragment amplified using the specific primer pair *P-fwr*-C A C A G C T G G G A A T G G A A G A T and *P-rev*-CCCTCAACCTTTGATGCAAT and labeled with digoxigenin-11-dUTP using DIG RNA Labeling Kit (SP6/T7) according to the manufacturer's protocol.

The amplified fragment was cloned in pCR[®] 2.1-TOPO TA vector (Invitrogen) and positive clones were selected to confirm probe sequence. The plasmid was linearized with *Hind*III (Fermentas) and the probe was generated using the T7 RNA polymerase in the presence of a digoxigenin-substituted nucleotide mix. A second probe was generated using the SP6 polymerase after linearization with *Eco*RI (Fermentas) and used as a control for in situ hybridization. Template DNA was removed using DNase I for 15 min at 37 °C and then purified with Micro Bio-Spin chromatography columns (Bio-Rad). Both sense and antisense probes (synthesized using the SP6 or T7 promoter, respectively) were quantified with a NanoDrop 2000c.

Northern Blot Analysis

Total RNA was obtained from *P. lividus* tube feet discs and the stems, and *Nectin* expression was investigated using a 472-bp DIG-RNA labeling probe using the DIG Northern Starter Kit (Roche) following the manufacturer's protocol. Briefly, 1 µg of RNA was size-fractionated on a MOPS/formaldehyde agarose gel at 1.0 %, capillary transferred overnight to a Hybond

N⁺ nylon membrane (Amersham), and then fixed to the membrane by cross-linking by baking for 30 min at 120 °C. The hybridization was performed overnight in a DIG Easy Hyb Buffer (Roche) with 100 ng/ml of denatured DIG-RNA labeling probe at 68 °C followed by two stringency washes at 65 °C. The immunological detection was performed with antidigoxigenin-AP at a dilution of 1:2000 and visualized with the colorimetric substrates NBT/BCIP.

Southern Blot Analysis

The southern blot was performed with a restriction enzyme-digested genomic DNA to determine the number of *Nectin* gene copies in a genome. Genomic DNA was extracted using phenol/chloroform/isoamyl alcohol protocol and 2.5 µg of total DNA was digested separately with *Ned*I, *Pvu*II, and *Msp*I restriction enzymes. The probe hybridization and detection were performed using Detection Starter Kit I (Roche) following the manufacturer's protocol. Briefly, digested DNA was size-fractionated on a 0.7 % agarose gel, then the gel was washed in 0.25 M HCl for 20 min at RT, rinsed with water, and treated twice with a denaturing solution (1.65 M NaCl, 0.5 M NaOH) for 20 min each. DNA was capillary transferred overnight to a Hybond N⁺ nylon membrane (Amersham), then washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and dried for 30 min at 80 °C. The 472-bp DIG-RNA labeling probe was used to detect DNA fragments. The hybridization was done at 65 °C overnight, followed by two washing steps at high stringency (65 °C). The immunological detection was performed with an antidigoxigenin-AP at a dilution of 1:2000 and visualized with the colorimetric substrates NBT/BCIP.

Quantitative real-time PCR

Quantitative real-time PCR was performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems), with a SYBR green fluorescent label. Before the reverse transcription for cDNA synthesis, the RNA was isolated using Trizol reagent and then purified with OneStep[™] PCR Inhibitor Removal Kit (Zymo Research) to the remove polyphenolic compounds and melanin. The expression of *Nectin-1* or *Nectin-2* was discriminated by RFLP before qRT-PCR analysis using a pool of cDNA from five individuals. The cDNA samples were diluted in a range of 100, 50, 25, 5, 1, and 0.25 ng and were used as RT-qPCR templates. The cDNA was mixed with 1× SYBR green master mix (Applied Biosystems) and 3–5 pmol of each primer. Samples were run in triplicate in 96-well plates (Corning, NY) and qRT-PCR data were analyzed with the Relative Manager Software (Applied Biosystems, USA) to estimate transcript levels of each sample using the $2^{-\Delta\Delta C_t}$ method according to the manufacturer's instructions. To find an adequate

endogenous control for qRT-PCR, three different housekeeping genes were tested: β -actin (NM_001037157); glyceraldehyde-3-phosphate dehydrogenase (KC775387); and ubiquitin (JQ793792). The primers used to amplify each gene and expected sizes of PCR products are indicated in Supplementary Table 2. Cycling parameters were as follows: 95 °C for 10 min, then 40 cycles at 95 °C for 15 s and 60 °C for 60 s. A melting temperature-determining dissociation step was performed at 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s at the end of the amplification phase. The standard curves were generated by plotting the log cDNA values against Cq values obtained over the range of dilutions. The pairwise variation of housekeeping genes was analyzed based on cycle threshold (Ct) values between the individual samples and the experimental conditions. The fold change in gene expression of target genes relative to the housekeeping genes was evaluated using $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). Results were analyzed and shown as fold change (log10 relative quantification) relative to the control. Data from biological replicates were averaged and shown as normalized gene expression \pm standard error of the mean (SEM). Statistical analysis was performed using Pearson linear correlation.

In Situ Hybridization

Whole-mount in situ hybridization (ISH) was performed according to Arendt et al.'s (2004) protocol, with slight modifications. Adult *P. lividus* attached to the bottom of an aquarium were forced to detach causing a number of tube feet to break by the stem. They were later collected, washed with 10 mM EDTA, and fixed for 4 h at room temperature (RT) in 4 % paraformaldehyde prepared in phosphate-buffered saline (0.2 M phosphate buffer, 0.1 M NaCl, pH 7.4 with 20 mM EDTA). Fixated tube feet were washed twice in PBS, incubated for 6 h in 12 % sucrose in PBS, embedded in Tissue Freezing Medium (Fisher Scientific), and then frozen in a super-cooled isopentane bath in dry ice. Tissue sections (12 μ m in thickness) were obtained using a freezing microtome and kept at -20 °C until use. ISH experiment was adapted from DIG Application Manual for in situ hybridization of Roche. Briefly, the sections were washed twice in PBS, incubated for 5 min in 10 mM glycine in PBS, permeabilized with 0.3 M of Triton in PBS for 15 min, and then with 2 μ g/ml of proteinase-K in PBS for 10 min at RT. Permeabilized sections were postfixed in 4 % paraformaldehyde for 10 min, washed in PBS, and then acetylated with 0.1 M RNase-free triethanolamine containing 0.25 % acetic anhydride (pH 8.0) for 10 min with stirring. The sections were washed with PBS and prehybridized for 2 h at 42 °C in 40 % deionized formamide, 10 % dextran sulfate, 10 mM DTT, 5 \times SSC, and 400 μ g/ml of yeast RNA. Tissue sections were then denatured for 5 min at 80 °C followed by hybridization at 42 °C with the same buffer plus 2 ng/ μ l of probe for 16 h. After

hybridization, the sections were washed twice in 2 \times SSC containing 50 % formamide at 37 °C for 15 min, then 2 min in 0.2 \times SSC at (RT) temperature, and finally with 100 mM Tris–HCl, containing 100 mM NaCl (TBS), pH 8.0, for 5 min at RT. Sections were then incubated with antidigoxigenin conjugated to alkaline phosphatase (Roche) diluted at 1:1000 in TBS containing 0.5 % of blocking reagent (Roche) for 2 h at RT. Excess antibody was removed by two washes with TBS for 15 min. Color was developed in the dark for 16 h at RT in TBS containing the colorimetric substrates NBT/BCIP (Roche). Staining was stopped by two washes in TBS and finally with distilled water. Sections were then equilibrated in 70 % glycerol for 2 h and mounted in mounting medium (Sigma) for inspection and photography.

Results

Nectin cDNA Analysis in Sea Urchin Tube Feet

The cDNA encoding *Nectin* was amplified for the first time from adult *P. lividus* sea urchin tube feet, showing that not only the egg/embryo *Nectin* (GenBank AJ578435) is expressed in these adhesive organs but also a new *Nectin* sequence. Therefore, these two sequences were named for simplicity, *Nectin-1* and *Nectin-2*, respectively. Similarly to the previously known *Nectin-1*, the amplified cDNA of the newly found *Nectin-2* has 3593 bp in length and contains a 2952-bp open reading frame encoding for 984 aa residues, comprising a 24 aa signal peptide, and 6 tandemly repeated discoidin-like domains (DS domain), also known as F5/8 type C domain, at aa positions 26 to 173, 185 to 332, 345 to 493, 523 to 654, 666 to 815, and 828 to 978 (Fig. 1). This new mRNA sequence was deposited at GenBank under the accession number KT351732. A multiple sequence alignment of the two *Nectin* nucleotide sequences revealed that the new *Nectin-2* (GenBank KT351732) has 33 single nucleotide substitutions relative to *Nectin-1* (GenBank AJ578435), of which 52 % were between nucleotides A/G. Of these nucleotide substitutions, 18 were synonymous modifications and only 15 were non-synonymous modifications (Table 1). Within these 15 non-synonymous modifications resulting in effective predicted amino acid changes, 7 were classified as conservative, given that the change was between amino acids with similar physicochemical properties. The remaining predicted amino acid changes (missense point substitutions) in *Nectin-2* were found not to coincide with the LDT motif, nor the sugar binding sites and cysteines in the 6 tandemly repeated DS domains, previously identified as key elements for the function and structure of *Nectin-1* (Costa et al 2010). In terms of posttranslational modifications (PTMs), the predicted sulfation site Tyr77, referenced as being important for proteins that go through the secretory pathway (Costa et al. 2010), is

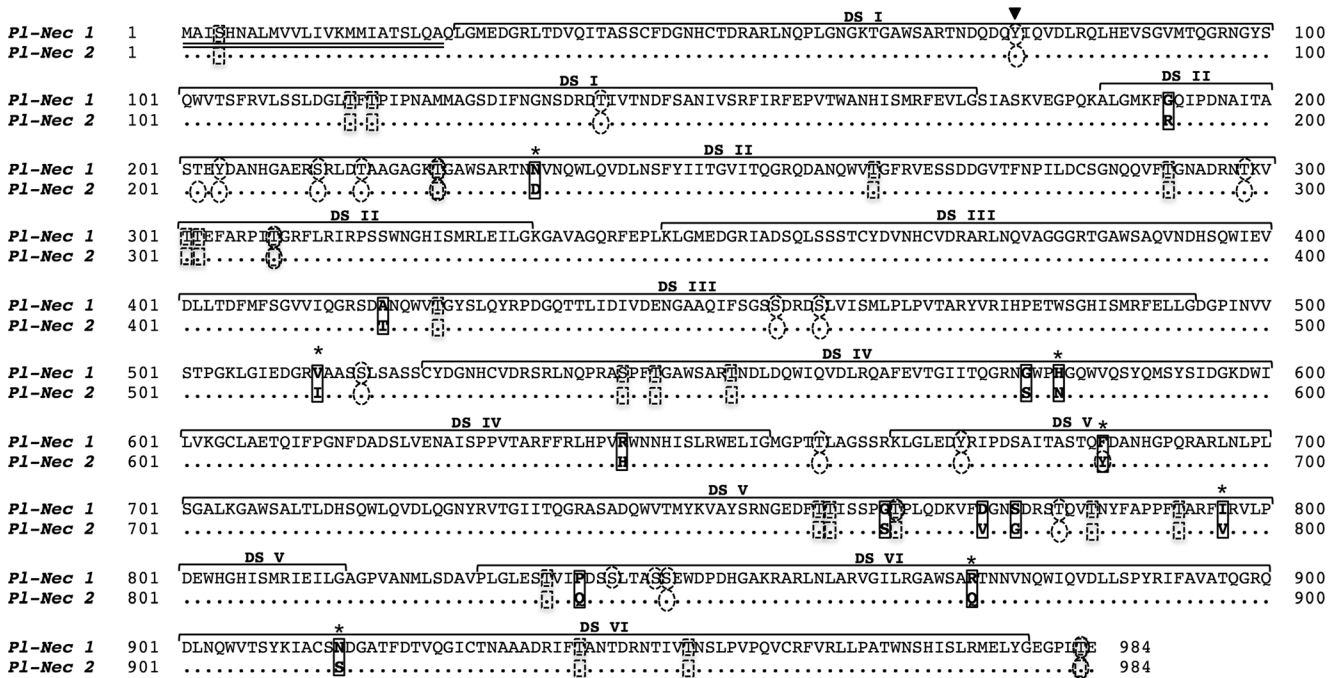


Fig. 1 Amino acid sequence alignment of *Paracentrotus lividus* *Nectin*. The differences between *Nectin-1* (GenBank AJ578435) and *Nectin-2* (GenBank KT351732) sequences, amplified from sea urchin adult tube feet, are highlighted in boxes; asterisks indicate conservative amino acid changes (between aa with similar physicochemical properties); double

underlines indicate the signal peptide; upper lines indicate the six tandemly repeated discoidin-like domains (DS); the arrow indicates the predicted sulfation site Tyr77; discontinuous circles indicate the predicted phosphorylation sites and discontinuous squares indicate the predicted glycosylation sites

also preserved in *Nectin-2*. In terms of phosphorylated sites, *Nectin-1* is predicted to be more prone to phosphorylation than *Nectin-2* is, the first presenting seven serine, nine threonine, and three tyrosine phosphorylated sites, whereas the second is predicted to have five serine, nine threonine, and four tyrosine phosphorylated sites (Fig. 1). In terms of glycosylation sites, both *Nectins* are predicted to have 2 serine and 20 threonine O-linked glycosylated residues (Fig. 1), but no N- or C-glycosites were predicted for any of the sequences.

In addition, PCR-RFLP was used as a fast screening method to identify and distinguish the two *Nectin* cDNAs amplified from sea urchin tube feet. Based on mRNA sequence analysis, the restriction enzyme *SsiI* was chosen for its ability to produce distinctive restriction fragment patterns for *Nectin-1* and *Nectin-2*. This allowed us to successfully amplify both *Nectins* and to discriminate them through the presence of two fragments of 318 and 440 bp specific for *Nectin-1* and one fragment of 758 bp specific for *Nectin-2* (Fig. 2). From a total of 20 individuals, *Nectin-2* was identified in 6 of them, constituting a point prevalence of 30 %, while *Nectin-1* was identified in the remaining 14, constituting a point of prevalence of 70 %.

Nectin Genomic DNA Analysis

Since nothing was known about *Nectin* genomic DNA, its sequence was obtained for the first time by primer

walking sequencing. We found that *Nectin* gene has 5644 bp, comprising five exons of 1025, 1596, 180, 161, and 633 bp in length, between nucleotides 1–1025, 1026–2620, 2621–2800, 2801–2061, and 2962–3593, respectively. Exon I coded for the first and second DS domains; exon II coded for the third, fourth, and fifth DS domains; and exons III and IV coded for the sixth DS domain. Exon I was flanked by typical splice signals 5' AG/GT and 3' CAG/NT but the other exons were different from the standard consensus (Fig. 3). This new genomic DNA sequence was deposited at GenBank under the accession number KT381970. The abovementioned nucleotide substitutions identified in the cDNA of the new *Nectin-2* were also present in the genomic DNA, indicating that these substitutions were not originated by mutations during transcription, such as alternative splicing or RNA editing. The Southern blot analysis reveals that the number of bands obtained after digestion with three different restriction enzymes corresponds to the size and number of predicted bands (Fig. 4). A single signal was obtained in the digestion with *NedI* and *PvuII* originating respectively one band with 10 kb and one band with 4.3 kb, while two signals were obtained in the digestion with *MspI* originating two bands with the predicted size of 1.2 and 1.7 kb. These results indicated that *Nectin* gene might be a single copy gene.

Table 1 Nucleotide point alterations observed in *Nectin B* (GenBank KT351732) relative to *Nectin A* sequence (GenBank AJ578435)

Nucleotide change (cDNA)	Amino acid change	Type of modification
c.213A>C	p.Thr71Thr	Silent
c.363A>G	p.Pro121Pro	Silent
c.411A<G	p.Arg137Arg	Silent
c.571G>A	p.Gly191Arg	Missense
c.582G>A	p.Ile194Ile	Silent
c.660T>C	p.Gly220Gly	Silent
c.697A>G	p.Asn233Asp	Missense
c.708G>A	p.Gln236Gln	Silent
c.1188G>A	p.Gln396Gln	Silent
c.1255G>A	p.Ala419Thr	Missense
c.1365T>C	p.Ser455Ser	Silent
c.1537G>A	p.Val513Ile	Missense
c.1578T>C	p.Gly526Gly	Silent
c.1716T>C	p.Arg572Arg	Silent
c.1732G>A	p.Gly578Ser	Missense
c.1741C>A	p.His581Asn	Missense
c.1922G>A	p.Arg641His	Missense
c.1938T>C	p.Ile646Ile	Silent
c.1983G>A	p.Ala661Ala	Silent
c.2054T>A	p.Phe685Tyr	Missense
c.2293G>A	p.Gly765Ser	Missense
c.2319T>C	p.Phe273Phe	Silent
c.2321A>T	p.Asp774Val	Missense
c.2329A>G	p.Ser777Gly	Missense
c.2386A>G	p.Ile796Val	Missense
c.2510C>A	p.Pro837Gln	Missense
c.2601C>T	p.Arg867Arg	Silent
c.2618G>A	p.Arg873Gln	Missense
c.2703T>C	p.Asp901Asp	Silent
c.2744A>G	p.Asn915Ser	Missense
c.2787C>T	p.Asn929Asn	Silent

These point alterations resulted in 18 synonymous (silent) and 15 non-synonymous (missense) substitutions

***Nectin* Comparative Expression Analysis in Tube Feet Discs and Stems**

In order to evaluate *Nectin* mRNA expression levels in tube feet discs (the distal and enlarged part of the tube feet used for substratum attachment) and stems (proximal cylindrical part of the tube feet used for movement), a semiquantitative RT-PCR was performed. For this, *Nectin* was coamplified with a housekeeping β -actin gene, revealing that *Nectin* is indeed overexpressed in adhesive tube feet discs in comparison with stems (Fig. 5a). These results are further corroborated by the Northern blot analysis using a specific labeling RNA-DIG probe that confirms *Nectin* mRNA overexpression in the

Restriction sites predicted in *Nectin* genes (enzyme *SsiI*)

<i>Nectin-1</i>	266, 653, 1541, 1981, 2299, 2600, 2810, 2823, 2868
<i>Nectin-2</i>	266, 653, 1541, 2299, 2810, 2823, 2868

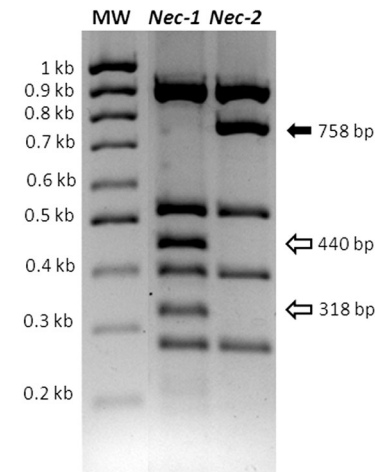


Fig. 2 Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified *Nectin* sequences with the restriction enzyme *SsiI*. The table indicates the restriction sites predicted for *Nectin-1* (GenBank AJ578435) and *Nectin-2* (GenBank KT351732). *MW*, molecular weight ladder, lane 1; digestion profile of *Nectin-1*, lane 2; digestion profile of *Nectin-2*, lane 3. White arrows highlight the 318 and 440-bp fragments specific for *Nectin-1*, while the black arrow highlights the 758-bp fragment specific for *Nectin-2*

adhesive tube feet discs relative to stems (Fig. 5b). Quantitative expression data for *Nectin* were afterward obtained by qRT-PCR, using a comparative Ct method ($\Delta\Delta Ct$). Among the three tested housekeeping genes, β -actin was selected as endogenous control for qRT-PCR analysis, given its high stability in terms of expression levels between the individual samples and experimental conditions. Using $\Delta\Delta Ct$ values to calculate fold differences in mRNA levels, a significant difference in the expression levels between tube feet discs and stems was observed in both *Nectin* mRNA sequences ($P < 0.05$) (Fig. 6). The expression of *Nectin-1* and *Nectin-2* were, respectively, 501- and 316-fold higher in the disc relative to the stem. No significant differences ($P = 0.1$) were observed between individuals expressing *Nectin-1* or *Nectin-2*. *Nectin* expression was also evaluated in tube feet from sea urchins just after their collection versus tube feet from sea urchins that were kept in aquarium. Expression analyses by coamplification of *Nectin* and β -actin, as well as the Northern blot assay reveals an overexpression of *Nectin* mRNA in tube feet discs from sea urchins just after collection from the field in comparison with tube feet discs from sea urchins that were acclimated to aquarium conditions (Fig. 7a, b). The qRT-PCR shows that the expression of *Nectin-2* is 1.5-fold higher ($P < 0.05$) in tube feet discs from sea urchins recently collected from the field in comparison with tube feet discs from sea urchin that were kept in aquarium. Unlike the disc, in tube feet stems, *Nectin* mRNA

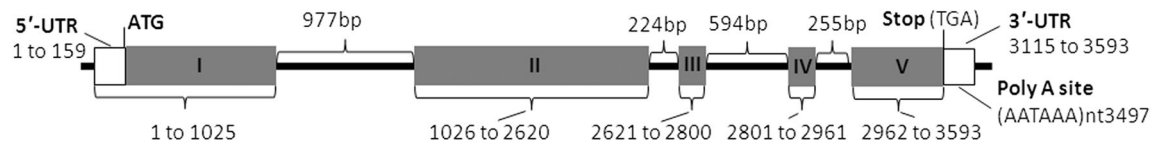


Fig. 3 *Paracentrotus lividus* *Nectin* genomic DNA sequence structure (GenBank KT381970). Numbers indicate the nucleotide positions in the mRNA sequences of both *Nectin-1* (GenBank AJ578435) and *Nectin-2*

(GenBank KT351732). The boxes represent the five exons (I–V) the gray ones are in the coding sequence (CDS) and the white ones in the untranslated regions (UTRs). The intron sizes are represented above the back line

expression does not vary significantly between recently field collected and aquarium sea urchins ($P=0.27$) (Fig. 7c).

Nectin in Situ Hybridization in Sea Urchin Tube Feet

Tissue and cellular expression of *Nectin* was also investigated by in situ hybridization, using a DIG-labeled RNA probe for a unique region of *Nectin* sequence. No significant signal above background levels was observed in control sections hybridized with sense probes (synthesized using the SP6 promoter) (Fig. 7a). The in situ hybridization experiments confirmed the Northern blot and qRT-PCR results, showing an intense *Nectin* expression in the tube feet disc. Extensive labeling

was observed stretching from the disc ossicles (rosette and frame) to the distal surface of the disc, appearing to mirror the localization of the epidermal adhesive secretory cells (Fig. 8b, c). In addition, *Nectin* probe also labeled the disc cuticle (Fig. 8c).

Discussion

The present work is a follow-up of a previous paper in which a protein highly homologous to *P. lividus* *Nectin* (GenBank AJ578435) that had only been reported for eggs and embryos of the same species (Matranga et al. 1992) was for the first time identified in adult tube feet discs (Santos et al. 2013). In eggs and embryos, *Nectin* is secreted during embryogenesis, becoming part of the embryonic extracellular matrix and significantly increasing the binding of dissociated cells to the substratum (Matranga et al. 1992). Given its adhesive function in eggs and embryos of the same species, it was hypothesized that it could also perform a similar role in adult adhesive organs for substratum attachment (Santos et al. 2013).

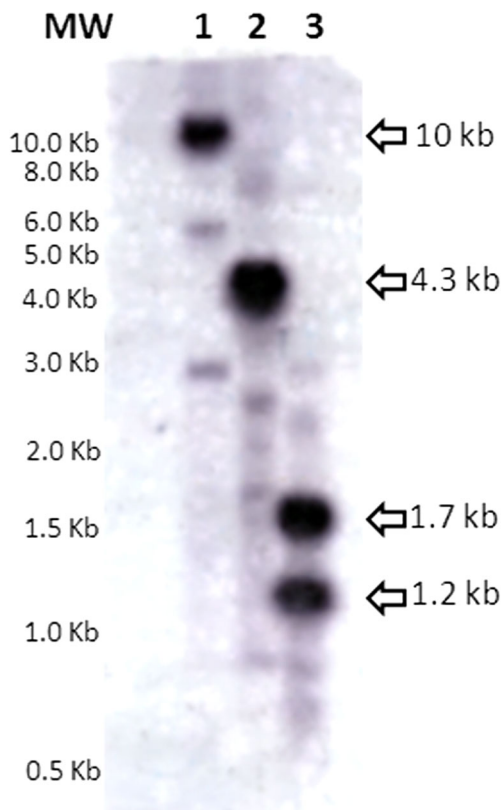


Fig. 4 Southern blot analysis of *Paracentrotus lividus* *Nectin* genomic DNA sequence. Genomic DNA was digested with the restriction enzymes *NdeI* (lane 1), *PvuII* (lane 2), and *MspI* (lane 3) and hybridized with a 472-bp DIG-RNA labeling probe. MW, molecular weight ladder; white arrows highlight the positive hybridization signals; numbers indicate the predicted size of DNA fragments

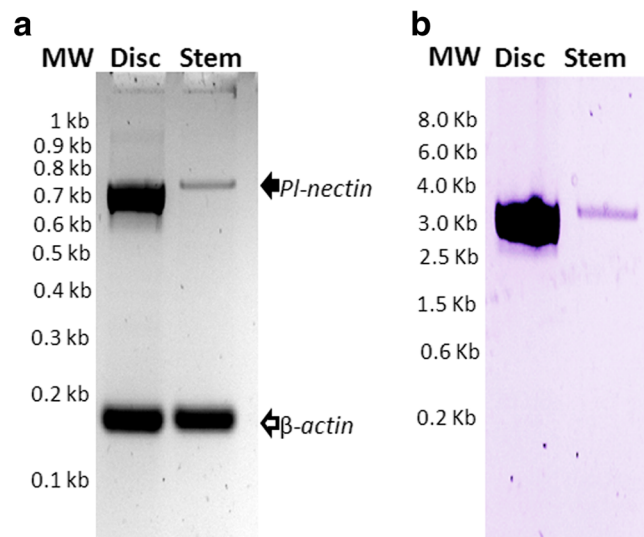


Fig. 5 *Nectin* mRNA expression levels in *Paracentrotus lividus* tube feet discs (adhesive part) and stems (non-adhesive part), assessed by semiquantitative RT-PCR and Northern blot. **a** RT-PCR performed using specific primers for *NecI* fragment (771 bp) coamplified with the house-keeping gene β -actin; **b** Northern-blot analyses performed using a 472-bp DIG-RNA labeling probe

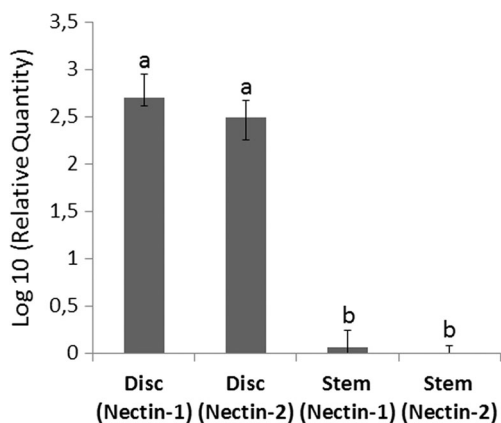


Fig. 6 Comparative expression analyses of *Nectin-1* (GenBank AJ578435) and *Nectin-2* (GenBank KT351732) genes in *Paracentrotus lividus* tube feet discs (adhesive part) and stems (non-adhesive part), assessed by qRT-PCR. The means of three independent experiments \pm SEM are shown. *P* values were determined with an unpaired *t* test; letters *a* and *b* indicate $P < 0.05$

Therefore, taking advantage of the peptide sequences previously obtained by MS/MS for the egg/embryo *Nectin* identified in adult tube feet discs by homology database search (Santos et al. 2013), primers were designed and the cDNA encoding for *Nectin* was for the first time amplified from adult *P. lividus* sea urchin adhesive organs. Our results show that *P. lividus* adult tube feet contain not only the cDNA sequence reported for eggs/embryos, called *Nectin-1* (GenBank AJ578435), but also a second cDNA sequence, called *Nectin-2*, differing in 33 single nucleotide substitutions, 52 % of which were between A/G nucleotides. Of these nucleotide substitutions, only 15 gave rise to effective amino acid changes (non-synonymous nucleotide substitutions), 7 of which were conservative amino acid changes (between amino acids with similar physicochemical properties). The new *Nectin-2* sequence was registered at GenBank under the accession number KT351732.

Further comparative sequence analysis of the new *Nectin-2* relative to the previously known *Nectin-1* showed that the predicted 15 amino acid changes do not alter the LDT motif, predicted to be the binding site to an $\alpha 4/\beta 7$ integrin receptor (Zito et al. 2010), nor the 6 tandemly repeated discoidin-like (DS) domains, predicted to bind molecules bearing galactose and N-acetylglucosamine carbohydrate moieties (Costa et al. 2010). Therefore, the new *Nectin B* is expected to maintain the key molecular elements identified as having a central role in *Nectin A* adhesive function, namely the LDT motif reported for its interaction with ectodermic cells and the DS domains reported for their interaction with carbohydrate molecules (Costa et al. 2010). Thus, in sea urchin adult tube feet, it can be hypothesized that the maintenance of these functional key elements in the expressed *Nectin-1* and *Nectin-2* are an indication that once secreted, (1) the LDT motif could have an adhesive role, bonding the adhesive secretion to the integrin

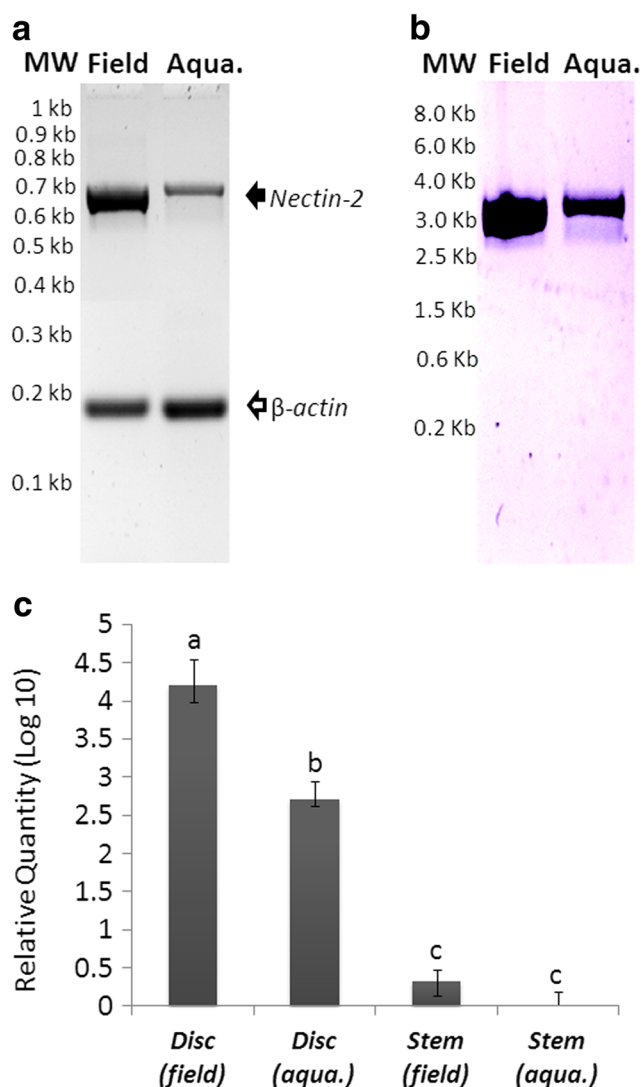


Fig. 7 *Nectin-2* mRNA expression levels in *Paracentrotus lividus* tube feet discs (adhesive part) and stems (non-adhesive part) from sea urchins just after collection from field versus sea urchins from aquarium, assessed by semiquantitative RT-PCR, Northern blot, and qRT-PCR. **a** RT-PCR performed using specific primers for *Nect1* fragment (771 bp) coamplified with housekeeping gene β -actin; **b** Northern blot analyses of *Nectin-2* using a 472-bp DIG-RNA labeling probe; **c** qRT-PCR of relative gene expression levels between tube feet discs and stems from sea urchins just after field collection versus sea urchins from aquarium. The means of three independent experiments \pm SEM are shown. *P* values were determined with an unpaired *t* test, letters *a* and *b* indicate $P < 0.05$ and *c* indicate $P = 0.27$

receptors in the disc epidermal cells (Zito et al. 2010) and (2) the DS motifs could have an adhesive role, bonding the adhesive secretion to the proteoglycans of the disc cuticle (Amey et al. 2000) as well as a cohesive role, bonding to the carbohydrate components of the adhesive (Santos et al. 2009). Furthermore, the new *Nectin-2* also preserves *Nectin-1* putative sulfation site at Tyr77 considered as an indication that the protein is secreted, providing additional evidence that both *Nectins* could be a component of *P. lividus* secreted adhesive.

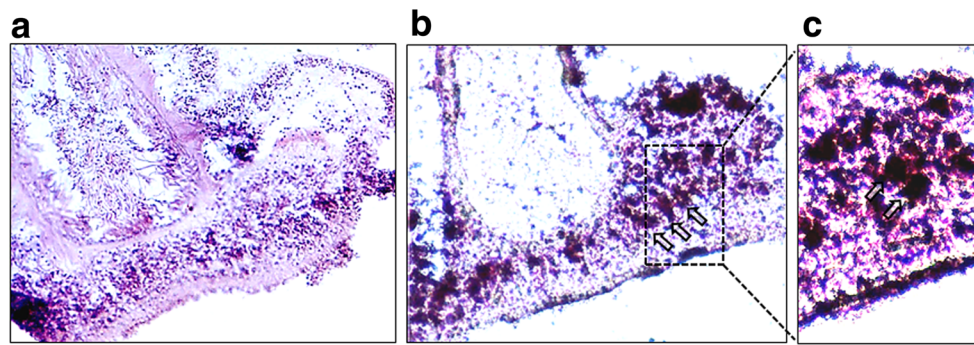


Fig. 8 *Nectin* mRNA expression levels in *Paracentrotus lividus* longitudinal tube feet cryosections assessed by in situ hybridization. Negative control (**a**) and in situ hybridization of adoral tube feet longitudinal cryosections labeled with a 472-bp DIG-RNA probe specific

for a unique region of *Nectin* (**b**, **c**). Abbreviations: AC, adhesive secretory cell; AE, adhesive epidermis; C, cuticle; CT, connective tissue; F, frame; LM, levator muscle; NE, non-adhesive epidermis; R, rosette; RM, retractor muscle

Nectin-2 also preserves *Nectin-1* cysteines in the DS domains, which are predicted to form intradomain S–S bridges being determinant for the protein structure (Costa et al. 2010). This is consistent with the reported insolubility of *P. lividus* secreted adhesive, its cysteine-biased content, and the presence of proteins in the adhesive containing intramolecular or intermolecular disulfide bonds (Santos et al. 2009). However, in terms of predicted phosphorylation sites, *Nectin-2* is predicted to have less phosphorylated serines (5 instead of 7) and more phosphorylated tyrosines (4 instead of 3) than *Nectin-1*. To date, there is no evidence of *Nectin* phosphorylation in eggs and embryos. However, in adult tube feet discs proteome, the identified *Nectin* was highly phosphorylated, forming a cluster of seven isoforms in 2-D gels, five of which were phosphorylated at different degrees (Santos et al. 2013). Although, there are no reports of the presence of phosphoproteins in *P. lividus* adhesive, its protein fraction presents a bias toward serine and threonine residues, making their adhesive proteins prone to phosphorylation (Santos et al. 2009). However, in other marine adhesive proteins, there are several reports of protein phosphorylation in serine residues (mussels, tube worms, and sea cucumbers) (Zhao et al. 2005; Flammang et al. 2009; Waite and Qin 2001). The role of phosphorylation in marine adhesives is not known, but it is believed to mediate adhesion to calcareous substrata (Waite and Qin 2001), plays a role in the condensation of the adhesive proteins in the adhesive cell secretory granules (Zhao et al. 2005), and/or may be involved in protein–protein cross-linking (Taylor and Wang 2007). Finally, in terms of glycosylation, both *Nectin-1* and *Nectin-2* are predicted to have 2 serine and 20 threonine O-glycosylation sites. Previous in silico analysis of *Nectin-1* sequence failed to predict any glycosylation sites (Costa et al. 2010), but in the tube feet disc proteome, five glycosylated *Nectin* isoforms were identified (Santos et al. 2013). Like phosphorylation, glycosylation is a recurrent PTM in marine adhesive proteins and N-, O- and C-linked glycoproteins have been reported in the adhesives secreted

by a wide range of marine organisms (green algae, gastropod mollusk, barnacle, mussel, and sea star) (Hennebert et al. 2011; Kamino et al. 2012; Ohkawa et al. 2004; Smith 2006; Stanley et al. 1999; Zhao et al. 2009). The roles of the sugar residues in marine adhesives are still unknown but are believed to increase conformational stability, enhance protein binding ability, and add resistance to enzymatic degradation (Roth et al. 2012; Zhao et al. 2009).

The existence of protein variants among marine adhesive proteins is not new. For example, the mussel *Mytilus edulis* presents several known adhesive foot protein variants namely from mfp-3 (>25 known variants), mfp-5 (2 known variants), and mfp-6 (5 known variants) (Yu et al. 2011). It has been proposed that having adhesive protein variants with varied isoelectric points and posttranslational modifications could increase the variety of interactions that these adhesives can undergo and thus provide flexibility to match diverse underwater surface features (Stewart et al. 2011; Zhao et al. 2006). The extensive variation in some mussel adhesive proteins has been attributed to a combination of allelic variation, conjugated with alternative splicing and RNA editing (Warner and Waite 1999).

In this study, *P. lividus Nectin* genomic DNA was obtained for the first time showing that it has 5644 bp in length and comprises five exons coding for the six DS domains. This new gene sequence was registered at GenBank under the accession KT381970. In addition, the 33 nucleotide substitutions identified in the cDNA of the new *Nectin B* were also found in *Nectin* genomic DNA, indicating that these substitutions were not originated by mutations during transcription, and thus alternative splicing or RNA editing events are most likely not the cause for the existence of multiple *Nectin* sequences. In addition, the Southern blot analysis using three restriction enzymes (*NedI*, *PvuII*, and *MspI*) resulted in a DNA band pattern consistent with the existence of a single copy of

Nectin gene. Taken together, our results demonstrate that in adult *P. lividus* tube feet, there are 2 mRNA variants of *Nectin* (*Nectin-1* GenBank AJ578435 and *Nectin-2* KT351732) differing in 15 missense nucleotide point substitutions that derive from a single gene. Therefore, it can be hypothesized that the existence of multiple *Nectin* sequences most likely derives from nucleotide substitutions (SNPs) during DNA replication due for example to high gene expression. Like suggested for mussels by Warner and Waite (1999), sea urchins are also subjected to high demand of adhesive proteins, particularly during periods of high water flow, which would require a high output gene expression to provide sufficient amounts of adhesive proteins for repeated and quick attachment.

To further substantiate *Nectin* involvement in sea urchin substratum, mRNA expression analysis was performed to compare *Nectin* levels in the tube feet disc, the part of the adhesive organ that makes contact with and adheres to the substratum through the secretion of an adhesive, versus the stem, the part allowing the tube feet to lengthen, flex, and retract (Santos and Flammang 2006). Our quantitative RT-PCR and Northern blot results clearly demonstrate that both *Nectin-1* and the new *Nectin-2* transcripts are significantly overexpressed in the adhesive part of the tube feet—the disc, relative to the non adhesive part—the stem, indicating that *Nectin* adhesive abilities are most likely being used by adult sea urchins for substratum attachment as proposed by Santos et al. (2013). These results are further corroborated by the demonstration that the expression of the two *Nectin* mRNA sequences is 1.5-fold increased in tube feet from sea urchins just after collection from field relative to sea urchin from aquarium, suggesting that *P. lividus* might be able to adjust the expression of *Nectin* to the hydrodynamic conditions. These results are in agreement with previous reports of a decrease in sea urchin tenacity (attachment force/unit area) of up to four times when comparing values measured just after collection from the field and values once the same individuals are maintained in aquarium (Santos and Flammang 2007). These results strongly suggest that sea urchins' ability to continuously adapt their attachment force to the hydrodynamic conditions is achieved by regulating the expression of *Nectin* mRNA and most likely concomitantly with the regulation of *Nectin* expression at the protein level. Further confirmation of *Nectin* involvement in sea urchin reversible adhesion was obtained by in situ transcript tissue localization, showing intense labeling in the disc adhesive epidermis and the disc cuticle. The obtained labeling mirrors the positioning of the epidermal adhesive secretory cells that deliver the adhesive secretion through the disc cuticle onto the surface, strongly binding the tube foot disc to the substratum (Flammang et al. 2005).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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