Molecular biology and genetics / Biologie et génétique moléculaires

A molecular study of Tunisian populations of *Dugesia sicula* (Plathelminthes, Tricladida) through an identification of a set of genes

Emna Meddeb [a,b,*], Mohamed Charni [c,d], Rim Ben Abdallah [a,c], Faten Raboudi [e], Sami Fattouch [a]

[a] *Laboratory of Food and Molecular Biochemistry, National Institute of Applied Sciences and Technology (INSAT), University of Carthage, Zone Urbaine Nord, 1080 Tunis, Tunisia*
[b] *Faculty of Sciences of Bizerte, University of Carthage, Tunis, Tunisia*
[c] *Faculty of Sciences of Tunis, University of Tunis El Manar, Tunis, Tunisia*
[d] *College of Sciences and Humanities of Dawadmi, Shaqra University, Shaqra, Kingdom of Saudi Arabia*
[e] *ISAJC, Bir El Bey, University of Tunis, Tunis, Tunisia*

A R T I C L E   I N F O

A B S T R A C T

Cell regeneration is a natural repair of different types of tissue after an injury or a lesion, and is associated with asexual reproduction in some animals such as planarians. Its understanding and improvement could have repercussions for tissue repair and regeneration as far as humans are concerned. In this context, we have proceeded to an essential step, which is the identification of the genes involved in planarian regeneration in the model species. *Dugesia sicula* Lepori (*D. sicula*) is distributed around the Mediterranean Sea, and this population is found in most of Tunisian dams. The collection of identified genes is already known in other species. DjFoxG, DjPC2, DjotxA, and Cathepsin-D were identified by the PCR technique and their expression was confirmed by RT-PCR and *in situ* hybridization. *DjFoxG* gene, the *FoxG1* homolog, is expressed throughout the planarian body, abundantly on stem cells. Consecutively, we choose a central nervous system (CNS) marker; the prohormone convertase 2 (*DjPC2*) gene. *DjotxA* was observed in the brain and especially in the region surrounding the eyes (visual cells). The regenerative cells of the gut of *D. sicula* were scored by the *Cathepsin-D* gene expression, which belongs to the aspartyl protease family. We found significant results through RT-PCR and In Situ Hybridization (ISH) techniques, confirming the expression of *DjFoxG*, *DjPC2*, *DjotxA* and *Cathepsin-D* genes in our specimens.

© 2019 Published by Elsevier Masson SAS on behalf of Académie des sciences.

1. Introduction

To understand the functioning of different phenomena in planarians, it is crucial to study, at first, the expression of several genes using classic but essential techniques such as PCR, RT-PCR, and in situ hybridization (ISH). The frequently used species were *Dugesia japonica*, *Dugesia tigrina* and *Schmidtea mediterranea*, but in our work we have focused on the Tunisian specimen *Dugesia sicula* Lepori, and therefore this extends molecular research. This work is considered as the mandatory stage maintained before carrying out specific studies on this selected species. Planarians are the best model to understand the

* Corresponding author at: National Institute of Applied Sciences and Technology (INSAT), Zone Urbaine Nord, University of Carthage, 1080 Tunis, Tunisia.
E-mail address: Emna.Meddeb@fsb.rnu.tn (E. Meddeb).

regeneration phenomenon that fascinates humans since decades. For this reason, we targeted stem cells, responsible factors of regenerative property in flatworms [1]. We choose *DjFoxG*, since it is the first gene found in planarian stem cells populations [2]. It belongs to the *Fox* gene family, whose name is *forkhead* box, and it is related to the *Drosophila* gene fork head, corresponding to transcription factors [3]. Then we targeted the central nervous system (CNS); there are several genes having major roles in the CNS, such as the neural and cervical ones, coding for protein receptors, hormones, peptides, and others [4]. The *DjPC2* gene was selected and it is a neuropeptide expressed profusely in the planarian central nervous system and previously found in the sensory and motor neurons clusters [5]. It is affiliated to the subtilisin/kexin family containing the precursor convertases, possessing activities in endocrine and neuroendocrine systems [6]. *DjotxA* is a good visual cells marker, it was expressed in the cephalic ganglion, but more medially in the termini of the visual axons in *D. japonica* [7]. Finally, we targeted the aspartyl protease *Cathepsin-D* gene to stain the digestive system of *D. sicula*. This protease acts in the process of hemoglobin digestion by schistosomes after blood feeding from humans [8], as it contributes to protein digestion in the gut of planarians [9].

2. Material and methods

2.1. Planarians

D. sicula specimens were provided by Dr. Mohamed Charni, as explained previously [10,11] and maintained as described by Molina et al. (2007) [12] as currently for *D. japonica* due to their phylogenetic rapprochement [13]. We denote the specimens collected from the Joumin dam by *s1* and from Boussadia by *s2*.

2.2. In silico sequences analysis

Several software tools have been exploited in our work. The alignment concerns only the species maintained in the laboratory, which are *S. mediterranea, D. japonica*, and *D. sicula* and it is based on their COI (cytochrome *c* oxidase subunit I) mitochondrial genes. We downloaded the nucleotide sequences of *Cathepsin-D, DjFoxG, DjotxA*, and *DjPC2* genes already identified in *D. japonica* due to its phylogenetic proximity with our species. To align the selected sequences, we used ClustalX, which also included phylogenetic analysis in coordination with Njplot to confirm this phylogeny [14]. We examined the percentage of homology between *Dug. japonica* and *Dug. sicula*

sequences through DNAMAN data [15]. The BLAST (Basic Local Alignment Search Tool) program, based on Gene-Bank, had also ensured alignment and analysis sequence similarities [16]. For the polymerase chain reaction (PCR), a silico simulation was carried out, and it is about OLIGO 7 [17]. The data obtained using the software also allowed us to design the primers.

2.3. Reverse transcriptase–PCR reaction

To start RNA extraction, we transferred five planarians from each specimen into 1.5-ml tubes. To lyse cells, we froze worms in liquid nitrogen for 5 to 10 s. After adding 400 μl of Trizol reagents and homogenizing worms, we appended 80 μl of chloroform. The mixture was centrifuged at 12,000 *g* for 15 min at 4 °C, then we collected the supernatant. For RNA precipitation, 200 μl of isopropanol were added and centrifuged for 10 min in the same conditions. We keep the pellet containing RNA, then we washed it with 1 ml of ethanol 75%. It was allowed to dry after centrifugation at 7500 *g* for 5 min at 4 °C, then dissolved in 20 μl of nuclease free water. Then, to synthesize cDNA, we added to the obtained RNA (2 μg) 1 μl of 50 μM oligo (dT)20 and nuclease-free water up to a total volume of 12 μl, then we incubated this synthesis reaction medium at 65 °C for 5 min. A mixture of reagents composed from 5x Reaction buffer, 10 mM dNTP Mix, Ribolock RNAase, an inhibitor (20 U/μl), a RevertAid H Minus M-Mulv and a Reverse Transcriptase (200 U/μl) in a total volume of 20 μl was added to the 12-μl synthesis reaction medium. It was incubated at 42 °C for 60 min, then at 70 °C for 5 min. After cDNA synthesis, a PCR reaction was prepared as described in Sandmann et al. (2011) [18]. The primers to amplify *DjFoxG, DjPC2, DjOtxA* and *Cathepsin-D* fragments were successively designed as displayed in Table 1.

PCR cycling conditions have been adjusted and each one had different annealing temperatures as follows: the first step was denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at different temperatures: 35 °C; 40 °C; 45 °C; 50 °C and 55 °C for 45 s, and elongation at 72 °C for 1 min with a final elongation at 72 °C for 10 min. The prepared reaction media were investigated by electrophoresis on a 1% agarose gel containing ethidium bromide. We needed between 30 to 50 ng of DNA in a total volume of 30 μl to achieve sequencing. The technological method was Sanger dideoxy sequencing.

2.4. In Situ Hybridization (ISH)

For in situ probe generation, we made a PCR amplification of template cDNA. Primers selected to get labeled

Table 1
Primers designed by the OLIGO 7 data for *DjFoxG, DjPC2, DjotxA*, and *Cathepsin-D* gene fragments.

Genes	Forward (5'-3')	Reverse (5'-3')
DjFoxG	TTGATAATGATGGCAATTCG	TTAATTTGCCTGTTGTTCC
DjPC2	ACACTGGACAAGCTGATGGA	AACGCCCTCTACAATTGCAC
DjotxA	GCAGTTGTTATGTAGCTGGCA	CGTGTTGATGCTGATGTCGA
Cathepsin-D	GGCTTATCCGTCCATTTCAGT	AAGAAGAAAATTGCGCCCAG

riboprobes were the same as for RT-PCR, but were associated with Dig-labeled probes: *DjFoxG forward*: 5'-taatacgactcactatagggTTGATAATGATGGCAATTCG-3; *DjFoxG reverse*: 5'-atttaggtgacactatagTTAATTTGCCTGT-TGTTCC-3'; *DjPC2 forward*: 5'-taatacgactcactatagggA-CACTGGACAAGCTGATGGA-3'; *DjPC2 reverse*: 5'-atttaggt-gacactatagAACGCCCTCTACAATTGCAC-3'; *DjotxA forward*: 5′-atttaggtgacactatag GCAGTTGTTATGTAGCTGGCA; *DjotxA reverse*: 5′-taatacgactcactataggg CGTGTTGATGCT-GATGTCGA; *Cathepsin-D forward*: 5'-aattaggtgacactata-gAAGAAGAAAATTGCGCCCAG-3'; *Cathepsin-D reverse*: 5'-taatacgactcactatagggACACTGGACAAGCTGATGGA-3'. To synthesize RNA probes in vitro, we used T7 polymerase and Digoxigenin (Dig) labeling reagents. *For Dig-labeling, we needed a total volume of 11 μl; we used 1 μg of purified template DNA, 2 μl of 10x NTP Dig, 4 μl of 5× transcription buffer for T7 (Kpnl), 1 μl of Protector RNase inhibitor (R1 Ribolock), and finally 2 μl of T7 polymerase. After mixing gently and centrifuging briefly, we incubated the solution for 2 h at 37 °C. Then we added 1 μl of DNase 1 and 1 μl DNAse buffer to remove the template DNA.* This mixture was incubated for 15 min at 37 °C, and finally we stopped the reaction by adding 2 μl of 0.2 M EDTA. After purification by RNeasy Kit (Qiagen), we added 25 μl of nuclease-free water and measured the RNA concentration.

In situ hybridization (ISH) was performed as mentioned previously [19]. We selected intact (not regenerating) *s1* and *s2 D. sicula* specimens; they were starved for one week before ISH inauguration. Briefly, in a first step, we used 2%HCL/5/8 Holtfreter, Canoy, MetOH, and 5%H₂O₂/MetOH as solutions to remove mucus, fix, and bleach planarians. Animals were selected (same length and size) in 2 ml of each solution. Hybridization is performed on the second day after rehydration by decreasing volumes of EtOH/5/8/ PBS, preheat hybridization using a TPBS solution, protease digestion using 20 μg of proteinase K in 1 ml of TPBS, post-fixation by 5/8 PBS, 4%PFA/5/8 Holtfreter and TEA (0.1 M), acetylation by acetic anhydride and PBST, and prehybri-dization by prehybridization-mix (−20 °C) [20]. On day 3, posthybridization (washing) was carried out using de-creasing volumes of formaldehyde (CH₂O), then 2x SSC/ 0.1% Triton-X, Buffer I (malic acid buffer, Triton 10%, and H₂O) and Buffer II (Buffer I, horse serum). For anti-body incubation, we added 1:2000 anti-DIG/Buffer II for 2 h, then washed planarians by Buffer I, keeping them overnight. On the last day, the color reaction take place in the dark, employing Buffer I, TMN, and 20 μl of NBT/BCIP ml TMN. To fix the color reaction, we used 1× PBS, 4%PFA/ PBS, increasing then decreasing volumes of EtOH/PBS successively. Images of ISH samples were observed using a Leica M165 FC stereomicroscope.

3. Results

3.1. Bioinformatic analysis

The *D. japonica* genome is phylogenetically closer to *D. sicula* than *S. mediterranea*. The analyses were confirmed in silico by a ClustalX and Njplot software combination by drawing a phylogenetic tree (Annex 1). We downloaded *DjFoxG*, Dj*PC2, DjotxA* and *Cathepsin-D* and genes nucleo-

tide sequences of *D. japonica* to treat and deduce primers, by OLIGO7 data, among which we selected the best couple for each gene. The choice is fixed after performing several analyses using the same piece of software. The designed forward and reverse primers were stable and temperature conditions were optimal for all genes. We carried out the alignment (Fig. 1) of *DjFoxG* (Fig. 1a), *DjPC2* (Fig. 1b), and *Cathepsin-D* (Fig. 1c) fragments, obtained from *D. sicula*, with selected partial sequences from *D. japonica*, by Clustalx and DNAMAN software combinations perfor-mance. We found similarity between the *D. sicula* and *D. japonica* selected sequences through the BLAST program too. The homology analyses achieved by the DNAMAN software for both species were close to those obtained by the BLAST program for both genes *DjFoxG* and *DjPC2*. Although there is a wide difference in *Cathepsin-D* genes homology results, the DNAMAN software shows about 53% between both species, whereas the BLAST program reveals 83% of homology. For the DjotxA gene, the PCR and RT-PCR reactions were not significant, so automatically it was not sequenced. The resulting PCR bands were sequenced and have been assigned under accession numbers in GenBank as displayed in Table 2. The accession numbers of their homologue sequences in *D. japonica* were mentioned in the same table.

3.2. Expression of the selected genes

The identified fragments were expressed by RT-PCR performance, except the DjotxA gene. The *DjFoxG* transcript had its highest expression under annealing at 35 °C, 40 °C and 45 °C, with a low expression at 50 °C and no expression at 55 °C. The *DjPC2* transcript had high expression under all chosen annealing temperatures. The *Cathepsin-D* transcript had low expression, except under annealing at 45 °C. We note the absence of *DjotxA* bands in all revelations. Different annealing temperatures were adapted to select the best commune degree. For sequencing as for hybridization, the PCR cycling program was moderate, with a best annealing temperature (45 °C) for all performed genes. Electrophore-sis analysis made on a 1% agarose gel of PCR reaction for sequencing reveals clear bands in expected size without nonspecific bands. We clean up the PCR product of *DjFoxG*, *DjPC2,* and *Cathepsin-D* with the Qiagen kit (Qiagen, Münster, Germany), then we measured their concentra-tions for sequencing; the final products have been treated to have an appropriate concentration of 50 ng. Even though there is no band for *DjotxA*, we tried anyway in situ hybridization. Contrary to what was expected in PCR amplification and sequencing results, ISH for *DjFoxG* gene provided a doubtful result for both specimens, despite the optimization conditions (Fig. 2), but significant results for *DjotxA*. Hybridizations for *DjPC2* and *Cathepsin-D* were successfully revealed. In Fig. 3, the expression of *DjPC2* in the brain was indicated by white arrows, whereas it was represented by green arrows in the pair of longitudinal nerve cords (VNC). Furthermore, the brain branches and cephalic ganglions were clearly stained, which explains that this neuropeptide plays an important role in this cerebral parts. These results prove the expression of *DjPC2* in the central nervous system (CNS) of the *D. sicula* flatworm
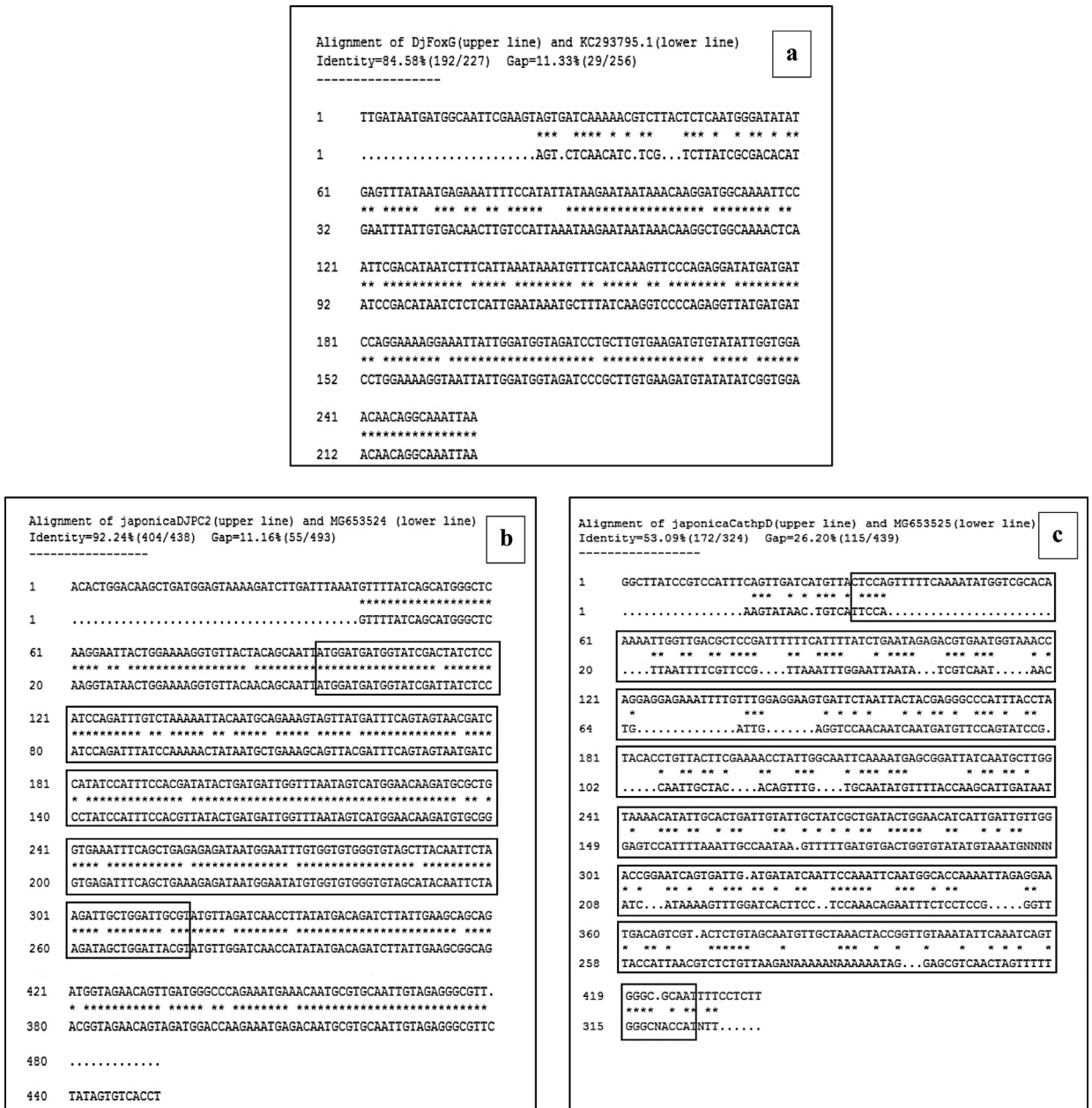
Fig. 1. Alignment of sequences by the DNAMAN software in combination with clustalX, which also reveals the percentage of homology between *Dugesia sicula* and *Dugesia japonica*. The framed sequences correspond to fragments that we identified in *D. sicula* (upper line): *DjFoxG* (KC293795.1); *DjPC2* (MG653524) and *Cathepsin-D* (MG653525). Alignment of *DjFoxG* amino acids fragments identified in *D. sicula* and already found in *D. japonica* (a). Alignment of *DjPC2* fragments identified in *Dug. sicula* and downloaded sequence from GenBank corresponding to *Dug. japonica* (b). Alignment of *Cathepsin-D* sequences in these species (c).

Table 2

GenBank accession numbers of the submitted *Dugesia sicula* nucleotide fragments and of their homologues already identified in *Dugesia japonica*.

GenBank accession numbers		
Nucleotide sequences	*Dugesia sicula*	*Dugesia japonica*
DjFoxG	KC293795	AB091061
DjPC2	MG653524	AK388877
Cathepsin-D	MG653525	AK389015

(Fig. 3). Otherwise, in situ hybridization results revealed that *DjotxA* is finally expressed (Fig. 4). For the *s1 D. sicula* specimen, *DjotxA* is clearly expressed in the visual cells, but is slightly stained in the brain (Fig. 4a) contrary to the *s2* specimen, in which *DjotxA* was abundantly expressed on the cephalic ganglion (Fig. 4b). *Cathepsin-D* is expressed in the *D. sicula* gut (Fig. 5). Accurately, this ventral view shows a clear staining of the flatworm digestive system, specifically the anterior branch, posterior trunks and diverticulum
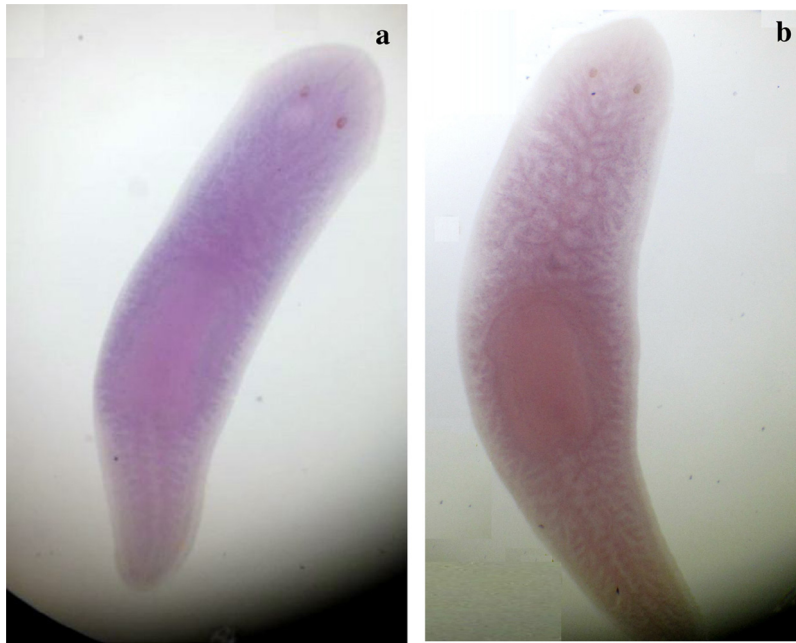
Fig. 2. Ventral view of *s1* and *s2* specimens of *Dugesia sicula* showing the in situ hybridization of DjFoxG. *s1* was a bit labeled but results could not be crucial (a). Results were not significant for the *s2 D. sicula* specimen (b).
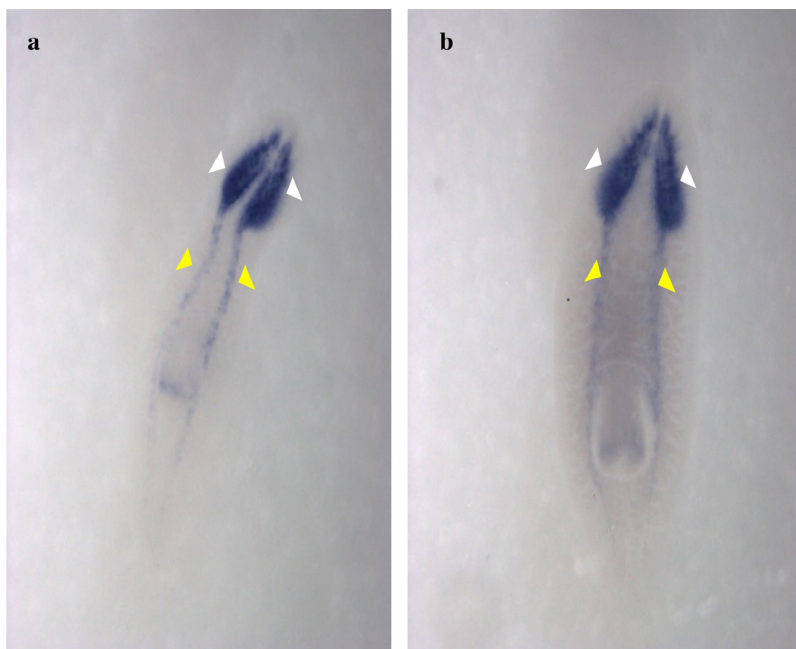

Fig. 3. Expression patterns of DjPC2 genes in *Dugesia sicula s1* (a) and *s2* (b) specimens. Ventral view of the in situ hybridization performance (a and b). *DjPC2* expression was detected on the central nervous system of *D. sicula*. It was expressed on the head (white arrows) and the ventral-dorsal nerve cords (yellow arrows).

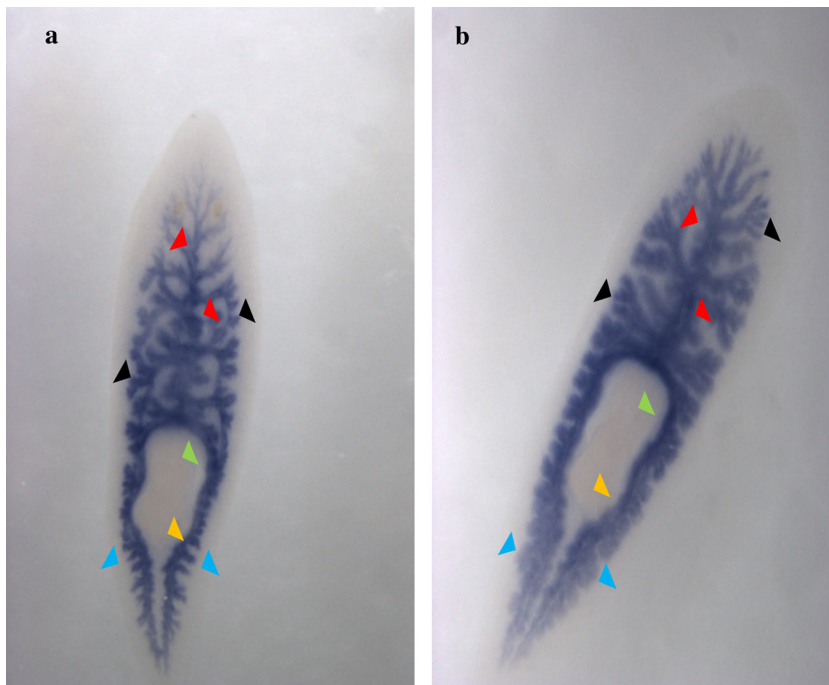of the gastrovascular cavity, except the pharynx and the mouth.

4. Discussion

The OLIGO7 database performs several analyses, which are essential to achieve the nucleotide sequences amplifi-cation selectively for best PCR results [17]. The primers size is usually between 20 and 30 nucleotides in order to ensure a sufficiently specific hybridization to RNA and cDNA sequences. They have to show the best possible comple-mentarities with both ends of the amplified sequence, so several criteria must be considered: for example, starting with ensuring the primers specificity by avoiding the
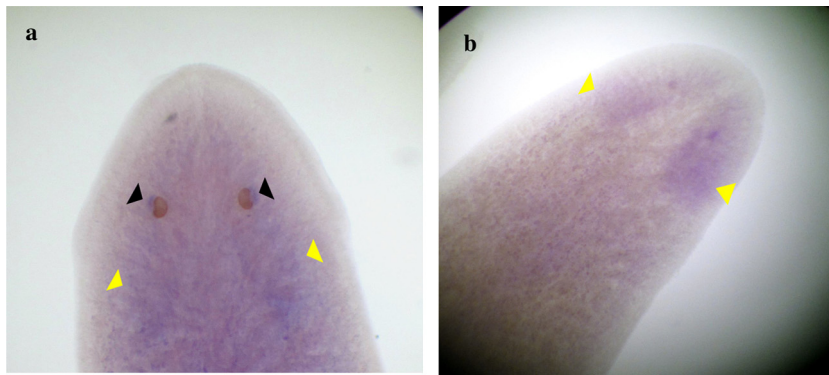
Fig. 4. Expression of *DjotxA* in *Dugesia sicula*. Specimen *s1* shows significant results, and the visual cells were clearly labeled (black arrows); we can almost discern expression in the brain (a). Expression of *DjotxA* in the head of specimen *s2* (cephalic ganglion) (b).

Fig. 5. Ventral view of the in situ hybridization revelation of the *Cathepsin-D* gene in *s1* (a) and *s2* (b) *Dugesia sicula* specimens. Expression of *Cathepsin-D* on the planarian's gut (a and b). Staining of the gastrovascular cavity in the anterior (red arrows) and posterior (blue arrows) parts of the flatworm with its diverticulum. Absence of staining in the pharynx (green arrow) and the mouth (orange arrow).

formation of binding sites and having minimum mismatches with all the sequences. Secondly, avoiding the pairing primers between each other (Duplex), and forming dimmers and hairpin loops that reduce the concentration of primers, by resulting secondary sites of fixation [21]. It is necessary that primers hybridize to the template cDNA under the same temperature conditions. Their stability is determined by the free energy ΔG (kcal/mol), which must be less negative, to avoid the fixation of primers on each other, and also to avoid their binding sites, bases mismatches, duplexes and hairpin formations [22]. Actually, we choose to amplify partial fragments from selected genes in order to get rid of ambiguous and stretched bases that ensure favorite conditions for the polymerase chain reaction. ClustalX, ensuring a progressive alignment, and the DNAMAN software for multiple sequence alignment, have been used

to confirm the species phylogeny rapprochement and to know the percentage of homology [16,23,24].

After bioinformatic analysis, expression was performed. Our RT-PCR results showed expression of all studied genes in *D. sicula* as predicted, except DjotxA, but these results confirm its genetic rapprochement to *D. japonica*. In situ hybridization for *DjFoxG* gene were blurred, so we cannot confirm, in a detailed way, the regions where it is normally expressed. Planarians were intact and this gene is a factor of transcription, highly expressed during regeneration [25]; this could be the reason of ISH expression deficiency. Recent studies confirmed that *DjFoxG* is a neural gene implicated in brain formation and that it is expressed in the cephalic ganglia in planarians [26]. *DjFoxG* had an expression in the body also, a strong one in the mesenchyme between epithelial layer and gut [28]. Its homolog *FoxG1* is a forkhead

gene specifically expressed in the telencephalon and retinal precursor cells in vertebrates [27] as mice [28]. It is expressed during otocyst development in zebrafish and mammals [29]. Then, for *DjPC2* (prohormone convertase 2), as previously demonstrated, it is a neuropeptide processing enzyme and it is known as a major neurotransmitter [10]. According to current research, it was expressed in the CNS during planarian regeneration [19]. The identified *DjPC2* in planarians has a high similarity with other animals [30]. It is known as a regulator in hypothalamus centers of the Siberian hamster [31]. Its homolog in mammals, which is the *PC2* gene, is expressed in the neuroendocrine tissue and it is responsible for neuropeptide proteolytic processing [32]. This gene mediates prohormones and proneuropeptides proteolytic maturation [33]. Only hybridization results for *DjotxA* were different between *D. sicula* species *s1* collected from Joumin and *s2* from Boussadia. This gene is normally expressed in both, brain and visual cells [7], and in a previous study it was restricted to a medial region of ganglia where they found the projection of the photoreceptor axons [34]. The *Cathepsin-D* gene was expressed as expected in *D. sicula*'s gut. It was known that aspartyl protease (*Cathepsin-D*) is localized in the digestive vacuolar compartments [35]. Previous research [36] confirms that a proteolytic cascade of this enzyme is used by the *Schistosoma mansori* parasitic worm to degrade hemoglobin into amino acids. Moreover, aspartyl protease has a blood-feeding function in the gut. This enzyme could have an important function in regeneration processing in planarians, while it was present during the cell differentiation stage in *D. tigrina* [37]. Interestingly, *Cathepsin-D* is synthesized from the midintestinal gland in response to viral infection [38]; so it contributes possibly in pathogens elimination [39].

In conclusion, these results confirm the current phylogenetic hypothesis and add prospects for initiating more appropriate studies concerning the species *D. sicula*. Bioinformatics pieces of software were fundamental to carry out molecular analysis as they facilitate the understanding of expression processing. The winged helix transcription factor *DjFoxG* was expressed through the RT-PCR technique, but unfortunately in this study the in situ hybridization (ISH) performance was low, but it could be definitively guaranteed in the future if we use *D. sicula* planarians that are in the process of regeneration. RT-PCR and ISH performances had shown a high expression of *DjPC2* and *Cathepsin-D*. The *DjotxA* expression noticed in our work was exactly in the same region, compared with the work by Umesono et al. (1999) [7], which was realized on *D. japonica*. According to the ISH performance of the central nervous system, digestive systems, visual and stem cells, it is clear that *D. sicula* specimens collected from different dams are anatomically slightly different, but are close to the species *D. japonica*, too [36,40]. We target a set of different genes because we believe that studying their expression in *D. sicula* is a basic, but useful work to carry out more developed research on this species and understand the function of some body regions, under well-defined biological conditions. Planarians have a remarkable regeneration processing ability and *D. sicula* could be a good model to extend molecular performance and enrich appropriate studies.

Appendix 1. Phylogenetic tree of COI mitochondrial genes corresponding to *Dugesia sicula* (DQ666035), *Dugesia japonica* (DQ666034), and *Schmidtea mediterranea* (AF178322) using Clustalx and NJPLOT combinations.
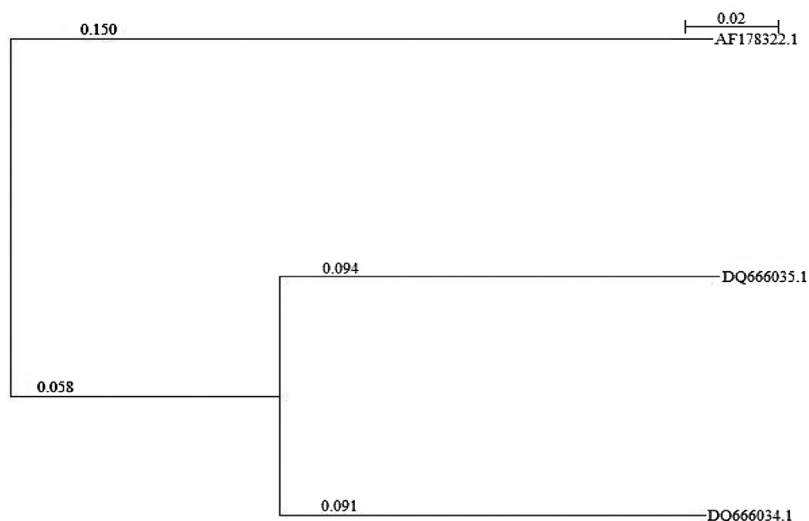
References

[1] E.M. Lázaro, M. Riutort, *Dugesia sicula* (Platyhelminthes, Tricladida): the colonizing success of an asexual Planarian, BMC Evol. Biol. 13 (2013) 268.

[2] K. Agata, Regeneration and gene regulation in planarians, Curr. Opin. Genet. Dev. 13 (5) (2003) 492–496. , http://dx.doi.org/10.1016/j.gde.2003.08.009.

[3] K.H. Kaestner, W. Knochel, D.E. Martínez, Unified nomenclature for the winged helix/forkhead transcription factors, Genes Dev. 14 (2) (2000) 142–146. , http://dx.doi.org/10.1101/gad.14.2.142.

[4] F. Cebria, T. Kudome, M. Nakazawa, K. Mineta, K.G. Ikeo, K. Agata, The expression of neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system, Mech. Dev. 116 (1–2) (2002) 199–204. , http://dx.doi.org/10.1016/S0925-4773(02)00134-X.

[5] T. Ouimet, A. Mammarbachib, T. Cloutier, N.G. Seidah, V.F. Castellucci, cDNA structure and in situ localization of the Aplysia calzfornica prohormone convertase PC2, FEBS Lett. 330 (3) (1993) 343–346. , http://dx.doi.org/10.1016/0014-5793(93)80901-6.

[6] N.G. Seidah, S. Benjannet, J. Hamelin, A.M. Mamarbachi, A. Basak, J. Marcinkiewicz, M. Mbikay, M. Chrétien, M. Marcinkiewicz, The Subtilisin/Kexin Family of Precursor Convertases: Emphasis on PC1, PC2/7B2, POMC and the Novel Enzyme SKI-1, Ann. N.Y. Acad. Sci. 885 (1999) 57–74. , http://dx.doi.org/10.1111/j.1749-6632.1999.tb08665.x.

[7] Y. Umesono, K. Watanabe, K. Agata, Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes, Dev. Genes Evol. 209 (1999) 31–39. , http://dx.doi.org/10.1007/s004270050.

[8] P.J. Brindley, B.H. Kalinna, J.Y.M. Wong, B.J. Bogitsh, L.T. King, D.J. Smyth, C.K. Verity, G. Abbenante, R.I. Brinkworth, D.P. Fairlie, M.L. Smythe, P.J. Milburn, H. Bielefeldt-Ohmann, Y. Zheng, D.P. McManus, Proteolysis of human hemoglobin by schistosome cathepsin D, Mol. Biochem. Parasitol. 112 (1) (2001) 103–112. , http://dx.doi.org/10.1016/S0166-6851(00)00351-0.

[9] L.S. Goupil, S.L. Ivry, I. Hsieh, B.M. Suzuki, C.S. Craik, A.J. O'Donoghue, J.H. McKerrow, Cysteine and Aspartyl Proteases Contribute to Protein Digestion in the Gut of Freshwater Planaria, PLoS Negl. Trop. Dis. 10 (8) (2016) e0004893, http://dx.doi.org/10.1371/journal.pntd.0004893.

[10] M. Charni, A.H. Harrath, R. Sluys, S. Tekaya, F. Zghal, The freshwater planarian Dugesia sicula Lepori, 1948 (Platyhelminthes, Tricladida) in Tunisia: ecology, karyology, and morphology, Hydrobiologia 517 (1–3) (2004) 161–170, http://dx.doi.org/10.1023/B:HYDR.0000027344.07747.f7.

[11] E. Meddeb, M. Charni, T. Ghazouani, A. Cozzolino, F. Fratianni, F. Raboudi, F. Nazzaro, S. Fattouch, Biochemical and Molecular Study of Carpobrotus edulis Bioactive Properties and Their Effects on *Dugesia sicula* (Turbellaria, Tricladida) Regeneration, Appl. Biochem. Biotechnol. 182 (3) (2017) 1131–1143. , http://dx.doi.org/10.1007/s12010-016-2387-y.

[12] M.D. Molina, E. Saló, F. Cebrià, The BMP pathway is essential for respecification and maintenance of the dorsoventral axis in regenerating and intact planarians, Dev. Biol. 311 (1) (2007) 79–94. , http://dx.doi.org/10.1016/j.ydbio.2007.08.019.

[13] P.M. Alvarez, J. Baguna, M. Riutort, Molecular phylogeny of land and freshwater planarians (Tricladida, Platyhelminthes): From freshwater to land and back, Mol. Phylogenet. Evol. 47 (2) (2008) 555–568. , http://dx.doi.org/10.1016/j.ympev.2008.01.032.

[14] M. McKenzie, M. Chiotis, C.A. Pinkert, I.A. Trounce, Functional Respiratory Chain Analyses in Murid Xenomitochondrial Cybrids Expose Coevolutionary Constraints of Cytochrome b and Nuclear Subunits of Complex III, Mol. Biol. Evol. 20 (7) (2003) 1117–1124. , http://dx.doi.org/10.1093/molbev/msg132.

[15] R. Du, R. Du, Y.F. Chen, X.R. An, X.Y. Yang, Y. Ma, L. Zhang, X.L. Yuan, L.M. Chen, J. Qin, Cloning and sequence analysis of myostatin promoter in sheep, DNA Seq. 16 (6) (2009) 412–417. , http://dx.doi.org/10.1080/10425170500226474.

[16] H. Yin, X. Zhao, Y.G. Du, Cloning and Molecular Characterization of a SKP1 Gene from *Brassica napus*, in: Proc. 3rd International Conference on Bioinformatics and Biomedical Engineering, iCBBE, 2009, p. 2009, http://dx.doi.org/10.1109/ICBBE.2009.5162512.

[17] W. Rychlik, OLIGO 7 Primer Analysis Software, In: Yuryev A. (ed.), PCR Primer Design, Methods Mol. Biol. 402 (2007) 35–59. , http://dx.doi.org/10.1007/978-1-59745-528-2_2.

[18] T. Sandmann, M.C. Vogg, S. Owlarn, M. Boutros, K. Bartscherer, The head-regeneration transcriptome of the planarian *Schmidtea mediterranea*, Genome Biol. 12 (8) (2011) R76, http://dx.doi.org/10.1186/gb-2011-12-8-r76.

[19] T. Nogi, M. Levin, Characterization of innexin gene expression and functional roles of gap-junctional communication in planarian regeneration, Dev. Biol. 287 (2) (2005) 314–335. , http://dx.doi.org/10.1016/j.ydbio.2005.09.002.

[20] M. März, F. Seebeck, K. Bartscherer, A Pitx transcription factor controls the establishment and maintenance of the serotonergic lineage in planarians, Development 140 (22) (2013) 4499–4509. , http://dx.doi.org/10.1242/dev.100081.

[21] O. Croce, F. Chevenet, C.R. Richard, OligoHeatMap (OHM): an online tool to estimate and display hybridizations of oligonucleotides onto DNA sequences, Nucleic Acids Res. 36 (2) (2008) 154–156. , http://dx.doi.org/10.1093/nar/gkn221.

[22] G.E. Tusnády, I. Simon, A. Váradi, T. Arányi, BiSearch:, primer-design and search tool for PCR on bisulfite-treated genomes, Nucleic Acids Res. 33 (1) (2005) e9, http://dx.doi.org/10.1093/nar/gni012.

[23] S. Iliyas, F.S. Sarkhawas, A Comparative Study of MSA Tools Based on Sequence Alignment Features and Platform Independency to Select the Appropriate Tool Desired, in: Proc. National Conference on Data Mining (NCDM 2011), 19–20th February 2011 (NCDM), Allana Institute of Management Sciences, Pune, India, 2011.

[24] P. Yin, J. Yan, R. Niu, C. Chen, J. Wang, J. Tan, Bioinformatics, Analysis of Chalconesynthase Gene in Morus, Agric. Sci. Technol. 14 (9) (2013) 1209–2011.

[25] S. Koinuma, Y. Umesono, K. Watanabe, K. Agata, The expression of planarian brain factor homologs, DjFoxG and DjFoxD, Gene Expr. Patterns 3 (1) (2003) 21–27. , http://dx.doi.org/10.1016/S1567-133X(02)00097-2.

[26] Z. Yuan, J. Zhang, B. Zhao, Z. Miao, X. Wu, Effects of perfluorooctanoic acid on neural genes expression and neuronal morphology in the planarian *Dugesia japonica*, Chem. Ecol. 32 (6) (2016) 575–582. , http://dx.doi.org/10.1080/02757540.2016.1162298.

[27] H.E. Moose, L.E. Kelly, S. Nekkalapudi, H.M. El-Hodiri, Ocular forkhead transcription factors: seeing eye to eye, Int. J. Dev. Biol. 53 (1) (2009) 29–36. , http://dx.doi.org/10.1387/ijdb.072505hm.

[28] J.M. Hébert, S.K. McConnell, Targeting of cre to the Foxg1 (BF-1) Locus Mediates loxP Recombination in the Telencephalon and Other Developing Head Structures, Dev. Biol. 222 (2) (2000) 296–306. , http://dx.doi.org/10.1006/dbio.2000.9732.

[29] S. Pauley, E. Lai, B. Fritzsch, Foxg1 is required for morphogenesis and histogenesis of the mammalian inner ear, Dev. Dyn. 235 (9) (2006) 2470–2482. , http://dx.doi.org/10.1002/dvdy.20839.

[30] K. Agata, Y. Soejima, K. Kato, C. Kobayashi, Y. Umesono, K. Watanabe, Structure of the planarian central nervous system (CNS) revealed by neuronal cell markers, Zool. Sci. 15 (3) (1998) 433–440. , http://dx.doi.org/10.2108/zsj.15.433.

[31] M. Helwig, R.M.H. Khooroshi, A. Tups, P. Barrett, Z.A. Archer, C. Exner, J. Rozman, L.J. Braulke, J.G. Mercer, M. Klingenspor, PC1/3 and PC2 Gene Expression and Post-Translational Endoproteolytic Pro-Opiomelanocortin Processing is Regulated by Photoperiod in the Seasonal Siberian Hamster (Phodopus sungorus), J. Neuroendocrinol. 18 (6) (2006) 413–425. , http://dx.doi.org/10.1111/j.1365-2826.2006.01431.x.

[32] S. Tanaka, Comparative Aspects of Intracellular Proteolytic Processing of Peptide Hormone Precursors: Studies of Proopiomelanocortin Processing, Zool. Sci. 20 (10) (2003) 1183–1198. , http://dx.doi.org/10.2108/zsj.20.1183.

[33] M. Mbikay, M.L. Raffin-Sanson, F. Sirois, L. Kalenga, M. Chretien, N.G. Seidah, Characterization of a repressor element in the promoter region of proprotein convertase 2 (PC2) gene, Mol. Brain Res. 102 (1–2) (2002) 35–47. , http://dx.doi.org/10.1016/S0169-328X(02)00180-8.

[34] E. Salò, D. Pineda, M. Marsal, J. Gonzalez, V. Gremigni, R. Batistoni, Genetic network of the eye in Platyhelminthes: expression and functional analysis of some players during planarian regeneration, Gene 287 (1–2) (2001) 67–74. https://www.sciencedirect.com/science/article/pii/S0378111901008630.

[35] B.J. Bogitsh, K.F. Krischner, *Schistosoma japonicum*: Ultrastructural localization of an hemoglobinase using mercury labeled pepstatin, Exp. Parasitol. 62 (2) (1986) 211–215. , http://dx.doi.org/10.1016/0014-4894(86)90025-1.

[36] P.L. Faust, S. Kornfeld, J.M. Chirgwin, Cloning and sequence analysis of cDNA for human cathepsin D, Proc. Natl. Acad. Sci. USA 82 (15) (1985) 4910–4914.

[37] F.B. Zamora-Veyl, H.L.M. Guedes, S. Giovanni-De-Simone, Aspartic Proteinase in *Dugesia tigrina* (Girard) Planaria, Z. Naturforsch. C 57 (5–6) (2002) 541–547.

[38] T. Rőszer, The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism, Cell Tissue Res. 358 (3) (2014) 685–695. , http://dx.doi.org/10.1007/s00441-014-1985-7.

[39] A. Venugopal, S.N. Kumar, Biochemical characterization of cathepsin D from the mussel *Lamellidens corrianus*, Comp. Biochem. Physiol. B 169 (2014) 25–30. , http://dx.doi.org/10.1016/j.cbpb.2013.12.003.

[40] Y. Umesono, K. Agata, Evolution and regeneration of the planarian central nervous system, Dev. Growth Differ. 51 (3) (2009) 185–195. , http://dx.doi.org/10.1111/j.1440-169X.2009.01099.x.