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ABSTRACT

Members of the *wnt* gene family encode secreted glycoproteins that mediate critical intercellular communications in metazoans. Large-scale genome and transcriptome analyses have shown that this family is composed of thirteen distinct subfamilies. They have further established that the number of *wnt* genes per subfamily varies significantly between metazoan phyla, highlighting that gene duplication and gene loss events have shaped the complements of *wnt* genes during evolution. In sea urchins, for example, previous work reported the absence of representatives of both the WNT2 and WNT11 subfamilies in two different species, *Paracentrotus lividus* and *Strongylocentrotus purpuratus*. Recently, however, we identified a gene encoding a WNT2 ortholog in *P. lividus* and, based on that finding, also reanalyzed the genome of *S. purpuratus*. Yet, we found no evidence of a *bona fide wnt2* gene in *S. purpuratus*. Furthermore, we established that the *P. lividus wnt2* gene is selectively expressed in vegetal tissues during embryogenesis, in a pattern that is similar, although not identical, to that of other *P. lividus wnt* genes. Taken together, by amending previous work on the *P. lividus wnt* complement, this work reveals an unexpected variation in the number of *wnt* genes between closely related sea urchin species.

Key words: Echinoderm; Sea urchin; *wnt2* ortholog; WNT ligand

Short title: A *Paracentrotus lividus wnt2* ortholog

INTRODUCTION

Signaling pathways mediated by WNT ligands are among the most important intercellular communication systems of metazoans (J. C. Croce & Holstein, 2014; Logan & Nusse, 2004). WNT ligands constitute a highly conserved family of secreted glycoproteins, which can be subdivided into thirteen distinct subfamilies. Importantly, the overall number of *wnt* genes per subfamily varies significantly between different metazoan phyla or even species (Janssen et al., 2010; Kusserow et al., 2005; Somorjai et al., 2018), suggesting that the *wnt* gene complements of extant animals resulted from complex superimpositions of gene duplication and gene loss events. In echinoderms, for example, the starfish *Acanthaster planci* has thirteen *wnt* genes, one per each subfamily (Yuan et al., 2019). In the two sea urchin species, *Paracentrotus lividus* and *Strongylocentrotus purpuratus*, however, the WNT2 and WNT11 subfamilies were reported missing (J. C. Croce et al., 2006; Robert et al., 2014). From these observations, it had hence been suggested that both *wnt2* and *wnt11* genes were present in the last common ancestor of echinoderms, but absent in the last common ancestor of *S. purpuratus* and *P. lividus* (Robert et al., 2014). Here, thanks to improved transcriptomic and genomic resources and improved analytical approaches, we report on the identification of a gene encoding a WNT2 ortholog in *P. lividus*, but not in *S. purpuratus*. Phylogenetic analyses further reliably place the newly identified *P. lividus* WNT sequence within the bilaterian WNT2 subfamily. In addition, we disclose the temporal and spatial expression patterns of the *P. lividus wnt2* gene throughout sea urchin embryogenesis.

RESULTS AND DISCUSSION

Identification of a *wnt2* ortholog in the sea urchin *Paracentrotus lividus*

In the course of a high-throughput transcriptome screen for genes regulated by TGFβ signaling pathways in *Paracentrotus lividus* (the results of which will be reported elsewhere), we identified a transcript (Pl_orfome15590) encoding a protein of 447 amino acids that displayed strong similarity to WNT ligands. The sequence of this protein differed from all WNT sequences previously described for this sea urchin

species (Robert et al., 2014). BLAST searches revealed that this unique sequence was most similar to WNT2 from other bilaterians. This result was intriguing, since a *wnt2* ortholog had not been described in previous surveys of the *wnt* gene complement of *P. lividus* (Robert et al., 2014) nor of that of a second sea urchin species *S. purpuratus* (J. C. Croce et al., 2006).

To confirm this finding, we hence reanalyzed the *P. lividus* genome, associated coding sequences, and protein prediction datasets using as query the sequence of the *Homo sapiens* WNT2 protein. These searches retrieved a number of *wnt* sequences that included the novel *wnt*-like sequence described above and which was missing in the previous report (Robert et al., 2014). To assess the orthology of this new WNT-like protein, we next conducted phylogenetic analyses. For this, we used both Maximum Likelihood (ML) and Bayesian Inference (BI). Both approaches confirmed that this new sequence encoded a WNT2 protein, placing it within the bilaterian WNT2 subfamily with strong statistical support (88% ML bootstrap support and 100% BI posterior probability) (Fig. 1; Suppl. Figs. 1, 2).

This discovery of a *P. lividus wnt2* gene is consistent with the presence of *wnt2* orthologs in several other echinoderms, such as the starfish *Acanthaster planci*, *Patiria miniata*, and *Patiria pectinifera* or the holothuroid *Apostichopus japonicus*, *Cladolabes schmeltzii*, and *Holothuria glaberrima* (Dolmatov, Afanasyev, & Boyko, 2018; Kawai, Kuraishi, & Kaneko, 2016; Mashanov, Zueva, & García-Arrarás, 2014; McCauley, Akyar, Filliger, & Hinman, 2013; Yuan et al., 2019). This finding further augments a previous report on the *wnt* complement of *P. lividus* (Robert et al., 2014), with this sea urchin species thereby possessing a total of twelve *wnt* genes, and not eleven.

Possible absence of a *wnt2* ortholog in the sea urchin *Strongylocentrotus purpuratus*

The discovery of a *wnt2* gene in *P. lividus* raised the question of the existence of this *wnt* gene in *S. purpuratus*. In this sea urchin species, a sequence annotated as a WNT2-like protein had previously been described (Sidow, 1992). However, when this sequence was included in our phylogenetic dataset, it did not branch with the bilaterian WNT2 subfamily, instead it associated with the WNTA subfamily with significant

statistical support (85% ML bootstrap support and 100% BI posterior probability) (Suppl. Figs. 3, 4).

To go further, we then carried out a new BLAST search and a HMMER analysis on all genomic and transcriptomic resources available for *S. purpuratus* on the EchinoBase website. As query for the BLAST search, we used the *P. lividus* WNT2 sequence, and as query for the HMMER analysis we employed a profile HMM that we built using the seven known echinoderm WNT2 sequences. Neither survey however identified a new *S. purpuratus wnt* sequence (data not shown).

Still not satisfied, we assessed the gene synteny conservation between the *P. lividus* scaffold containing the *wnt2* gene and the *S. purpuratus* genome. For this, we identified the ten genes upstream and the ten genes downstream of the *P. lividus wnt2* gene (Suppl. Fig. 5A) and characterized their *S. purpuratus* orthologs using BLAST searches (Suppl. Fig. 5B). Then, we analyzed whether these *S. purpuratus* sequences were located on identical scaffolds (Suppl. Fig. 5B) and whether a WNT-like sequence was encoded in their vicinity (Suppl. Fig. 5C). Strikingly, these analyses revealed that the *S. purpuratus* sequences corresponding to the ten genes upstream and the ten genes downstream of the *P. lividus wnt2* gene were located on several different scaffolds of the *S. purpuratus* genome (Suppl. Fig. 5B). Even though this analysis was made on an updated version of the *S. purpuratus* genome, it is still impossible to conclude whether this observed absence of gene synteny conservation results from real structural differences between the genomes of the two sea urchin species or whether it is due to technical issues associated with the genome assembly.

While analyzing the sequences of the identified *S. purpuratus* scaffolds, we nonetheless identified, on scaffold 1005, a DNA sequence that can be translated into a peptide (QTRHQCKCKHHW) (Suppl. Fig. 5C) featuring a WNT-like protein signature (Suppl. Fig. 5D). When this sequence was used as query for BLAST searches against the NCBI nr database, the best hits included several WNT proteins with very high e-values (above 200). Of note, no trace of this peptide was however found in any of the EST or transcriptome databases available for *S. purpuratus* on the EchinoBase website. Thus, at present, this sequence in the *S. purpuratus* genome (i) could result from genomic rearrangements or have been created during genome assemblies and is thereby artifactual, (ii) a *S. purpuratus wnt2* ortholog exists, but could not be detected due to

technical issues, or (iii) this peptide represents the remnant of an ancestral echinoid *wnt* gene, which, based on its genomic environment, might correspond to the lost *wnt2* gene.

Taken together, although we cannot formally rule out the presence of a *wnt2* ortholog in *S. purpuratus*, our data nonetheless suggest that the genome of the last common ancestor of echinoids encoded a *wnt2* gene, which may have subsequently been lost in some echinoid species, including *S. purpuratus*.

The temporal expression profile of *P. lividus wnt2*

To characterize the temporal expression profile of *P. lividus wnt2* during embryogenesis, we next carried out Northern blot and quantitative PCR (qPCR) analyses (Fig. 2). For both approaches, we surveyed the first 48 hours of development, i.e. from fertilized egg to pluteus larva. Both analyses yielded similar results, with expression of *P. lividus wnt2* becoming detectable at the mid-blastula stage, reaching a peak after hatching at the swimming blastula stage, and decreasing thereafter until the pluteus larva stage.

This expression profile is similar to that of the starfish *P. miniata wnt2* (McCauley et al., 2013). In addition, when comparing the expression levels of *wnt2* with that of other *P. lividus wnt* genes, *P. lividus wnt2* was among the most highly expressed *wnt* genes, with its maximum transcription level reaching that of *wnt8* (Robert et al., 2014). Last, *wnt2* is one of a number of *wnt* genes that start to be expressed at the blastula stage (along with *P. lividus wnt1, wnt4, wnt5*, and *wnt16*).

Given the temporal expression profile of *P. lividus wnt2*, the ligand encoded by this gene could interact with any of the four *P. lividus* FRIZZLED receptors, as all four are expressed when WNT2 proteins are presumably present (Robert et al., 2014). Therefore, *wnt2* could likely contribute to the implementation of one or more of several known WNT signaling-dependent developmental processes, including cell fate specification and coordination of morphogenetic movements.

The spatial expression profile of *P. lividus wnt2*

The spatial distribution of *P. lividus wnt2* transcripts was analyzed by whole-mount *in situ* hybridization assays (Fig. 3), carried out at the same developmental stages

surveyed by Northern blot and qPCR. Expression of *P. lividus wnt2* was not detectable in embryos prior to the late blastula stage (i.e. in fertilized eggs, 60-cell stage, and early- or mid-blastula stage embryos) (data not shown). At the late blastula stage, about one hour before hatching, *wnt2* was detected in a ring of cells located in the vegetal hemisphere (Fig. 3A,B). This expression persisted during blastula stages, up until the mesenchyme blastula stage (Fig. 3C-E). At the early gastrula stage, *wnt2* transcripts were located at the vicinity of the blastopore (Fig. 3F,G). At this stage, *wnt2* expression further became asymmetric along the aboral-oral axis, also referred to as the dorsal-ventral axis by some authors (Lapraz, Besnardeau, & Lepage, 2009). The expression of *wnt2* was indeed more conspicuous on the oral side. Then, as invagination proceeded, expression of *wnt2* persisted in the vicinity of the blastopore lips (Fig. 3H). During this process, since endoderm cells merge toward the blastopore and move through it to invaginate (Kominami & Takata, 2004; Martik & McClay, 2017), the expression of *wnt2* in any given endoderm cell must have been transient. Other genes transcribed around the sea urchin blastopore during archenteron development, such as *brachyury* (J. Croce, Lhomond, & Gache, 2001), also exhibit the same dynamic expression pattern. Moreover, during this process, the expression of *wnt2* continued to decline on the aboral side, while it remained conspicuous in the oral blastopore lip (Fig. 3H). Finally, at the prism stage, the *wnt2* transcripts were detectable only in the oral blastopore lip, the two oral arm buds, and the adjacent ectoderm (Fig. 3I,J).

In echinoderms, *in situ* hybridization patterns for a *wnt2* gene, during embryogenesis, have so far only been described in the starfish *P. miniata*. There, *wnt2* is expressed at the tip of the archenteron during gastrulation (McCauley et al., 2013). Like several other *P. lividus wnt* genes expressed at blastula stages, early *wnt2* expression surrounds the vegetal pole (Robert et al., 2014). Intriguingly, its subsequent expression, on the oral side of the blastopore, is the opposite of the pattern of *wnt5*, which is expressed in the corresponding aboral blastopore territories (Robert et al., 2014). Of note, in the sea urchin *Lytechinus variegatus*, *wnt5* has been proposed to control the specification of the border ectoderm and to be essential for skeletogenesis (McIntyre, Seay, Croce, & McClay, 2013).

In sum, although the functions of *P. lividus wnt2* remain unknown, its presence in the genome and its expression in restricted areas during morphogenesis suggest that WNT2-dependent signaling contributes to important steps in echinoderm development.

Perturbation studies will be essential to reveal those functions and the position of this gene within the gene regulatory networks that control endomesoderm and ectoderm patterning in sea urchins. In addition, it will be interesting to learn how *S. purpuratus* accomplishes those same functions, perhaps without a *wnt2* gene.

METHODS

Animals and embryo cultures

Adult *P. lividus* were collected in the bay of Villefranche-sur-Mer (France) and were kept at 16°C. Embryos were reared at 18°C (Lepage & Gache, 1989) until the appropriate developmental stages.

RNA isolation, cDNA library construction, and RNA sequencing

Embryos were collected at the late blastula stage and RNA was extracted using Trizol (Sigma-Aldrich). Polyadenylated transcripts were purified by two rounds of oligo-dT chromatography. cDNAs were synthesized with a Superscript double stranded cDNA synthesis kit (Invitrogen), fragmented by sonication, and cDNA fragments of 200-500 bp were gel purified. After addition of Illumina adapters to these fragments, the cDNA libraries were sequenced by the National Center of Sequencing (Genoscope, Institut de biologie François Jacob, Evry, France) on an Illumina HiSeq 2000 platform with 2x100bp paired end sequencing, thereby generating a pool of RNA reads. After sequencing, adapter sequences were removed and low quality reads were eliminated. In parallel, a reference transcriptome was obtained using Oases 0.2 (Schulz, Zerbino, Vingron, & Birney, 2012) by assembling reads generated by sequencing libraries constructed from a mix of embryonic and larval stages (unfertilized egg, 16-cell, early blastula, swimming blastula, mesenchyme blastula, early gastrula, late gastrula, prism, pluteus at 48h, 6-arm pluteus, 8-arm pluteus). The open reading frames of this reference transcriptome were extracted to generate a reference ORFome. The trimmed and clean RNA reads were mapped against this reference ORFome using Bowtie1 0.12 (Langmead, Trapnell, Pop, & Salzberg, 2009). Transcript counts were normalized and differentially expressed genes were identified.

Genome-wide identification of WNT proteins

The coding sequence of *P. lividus wnt2* was retrieved by BLAST searches conducted on the predicted coding sequence and protein datasets available for *P. lividus* on the Octopus web portal (http://octopus.obs-vlfr.fr/) using as query the *Homo sapiens* WNT2 sequence (GenBank accession number: NP_003382.1). Retrieved sequences were compared by BLAST2seq to previously reported *P. lividus* WNT sequences (Robert et al., 2014).

To identify a potential *S. purpuratus* WNT2 sequence, all genomic and transcriptomic datasets available for *S. purpuratus* on the EchinoBase website (http://www.echinobase.org/Echinobase/) were queried by BLAST using the *P. lividus* WNT2 sequence (GenBank accession number: MH427202). The e-value parameter was set to 10 to be as inclusive as possible. Retrieved sequences were verified by BLAST against the NCBI nr database and the *S. purpuratus* WNT sequences previously reported (J. C. Croce et al., 2006).

HMMER analysis

HMMER analyses are based on profile Hidden Markov Models (HMM) to establish sequence orthologies (Krogh, Brown, Mian, Sjölander, & Haussler, 1994). Using the seven currently available WNT2 sequences from echinoderms, an echinoderm WNT2-related profile HMM was generated using HMMER 3.2.1 (Eddy, 2011). This profile was employed as query against the *S. purpuratus* genomic and transcriptomic datasets available on the EchinoBase website (http://www.echinobase.org/Echinobase/). This analysis was carried out with the e-value parameter set to 10 to be as inclusive as possible. Retrieved sequences were verified by BLAST against the NCBI nr database and the *S. purpuratus* WNT sequences previously reported (J. C. Croce et al., 2006).

Phylogenetic analyses

Accession numbers of all the sequences included in our phylogenetic analyses are available in Supplementary Table 1. Multiple sequence alignments (available on request) were performed using MAFFT 7.397 (Katoh, Misawa, Kuma, & Miyata, 2002) with the EINSI parameters. To improve the analyses, poorly aligned protein regions (with more than 75% gaps) were deleted using TrimAl 1.4.1 (Capella-Gutiérrez, Silla-Martínez, & Gabaldón, 2009). Maximum-Likelihood (ML) phylogenies were calculated using IQTREE 1.6.2 (Nguyen, Schmidt, von Haeseler, & Minh, 2015) with the LG+G+I

substitution model. Branch supports were assessed based on 1,000 bootstrap pseudoreplicates. For Bayesian Inference (BI) phylogenies, two independent chains were run simultaneously using Phylobayes 4.1c (Lartillot, Lepage, & Blanquart, 2009) with the LG+G substitution model for 10,000 cycles. We assessed convergence using the MaxDiff parameter (MaxDiff < 0.1). A consensus topology was built by sampling one tree every 100th cycle and discarding the first 10% of the cycles. For readability, the ML and BI files were edited in FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and color-coded in Adobe Illustrator CS5 (Adobe Systems Incorporated). For ML, bootstrap values above 75 are considered as statistically significant support values for a given branch. The same applies for BI posterior probabilities above 0.95.

Gene synteny

On the *P. lividus* scaffold including *wnt2* (Pl_Scaffold_2100), we selected the ten genes located directly upstream and the ten genes located directly downstream of *wnt2*, and exhibiting an orthology with a predicted protein from *S. purpuratus*. A *S. purpuratus* ortholog of a given *P. lividus* sequence was defined as the BLAST best hit when querying, under default parameters, with the *P. lividus* sequence, the *S. purpuratus* peptide dataset available on the Echinobase website (http://www.echinobase.org/Echinobase/). Subsequently, the genome scaffolds containing these *S. purpuratus* orthologs were retrieved from the data available on the Echinobase website. A total of fourteen *S. purpuratus* scaffolds were collected and subsequently analyzed for gene content. Searches on these scaffolds for WNT proteins and WNT protein signatures, as defined by previously published WNT alignments (e.g. Bazan, Janda, & Garcia, 2012), were conducted by tBLASTn with the e-value parameter set to 10 to be as inclusive as possible. Retrieved sequence fragments were verified by BLASTx analyses against the NCBI nr database and the *S. purpuratus* WNT sequences previously reported (J. C. Croce et al., 2006).

Temporal expression profile

The Northern blot was carried out as previously described (Jenifer Croce, Lhomond, & Gache, 2003). The probe corresponded to the full-length cDNA of *P. lividus wnt2*, thereby including the entire open reading frame and some of the 5'-UTR region.

The qPCR analyses were also performed as previously described (Robert et al., 2014). The primer pair used for *P. lividus wnt2* was: 5'-GCACGCCTGATAGAGACACA-3' (forward), 5'-TCGAAGACTCCACCAACCTC-3' (reverse). The internal reference gene was *β-tubulin* and the primer pair used for this gene was: 5'-AGCTGACCACACCCACCTAC-3' (forward), 5'-ACGGTATTGCTGGCTACCAC-3' (reverse). The relative expression levels of *P. lividus wnt2* are provided relative to the level of expression of *β-tubulin*, set to 1,000. *P. lividus wnt2* expression levels were calculated using a classical comparative Ct method ($E^{-(Ct_{wnt2}-Ct_{beta\text{-}tubulin})}$) with the coefficient efficiency factor E = 1.94. In Figure 2, the qPCR analysis is reported as mean values obtained from three independent experiments with standard deviations of the means.

Spatial expression profile

Whole-mount *in situ* hybridization assays were carried out as indicated in Robert et al., 2014. The probe used for *P. lividus wnt2* corresponded to the full-length cDNA and was used at a final concentration of 1 ng/µl. Imaging of the stained specimens was performed using a Zeiss Axio Imager A2 microscope with differential interference contrast (DIC) optics.

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FIGURE LEGENDS

Figure. 1. The *Paracentrotus lividus wnt* complement. Unrooted phylogenetic tree reporting on the distribution of *P. lividus* WNT ligands in regards to the bilaterian WNT subfamilies. The topology of the tree is the one obtained by Maximum-Likelihood. Branch-lengths correspond to the number of substitution per site. Numbers at the nodes of each subfamily correspond to branch support from both Maximum-Likelihood and Bayesian Inference analyses (i.e. bootstrap values/posterior probabilities). Support values obtained for all branches from both analyses are provided in Supplementary Figures 1 and 2. Accession numbers of all the WNT protein sequences used in this analysis are available in Supplementary Table 1. *P. lividus* WNT proteins are highlighted in red and the *P. lividus* WNT2 protein is further pinpointed by a red arrow.

Figure. 2. Temporal expression profile of *Paracentrotus lividus wnt2* established by Northern blot (A) and quantitative PCR (B) analyses. Developmental stage abbreviations are as follows: E, Egg: fertilized eggs; 60, 60-cell: 60-cell stage; EB, E.blast: early blastula; MiB, M.blast: mid-blastula; LB, L.blast: late blastula; HB: hatched blastula; SB: swimming blastula; MB: mesenchyme blastula; EG: early gastrula; LG: late gastrula; Pr, Prism: prism stage; Pl, Plut.: pluteus larva. In (B), the relative level of expression of *P. lividus wnt2* was calculated relative to that of the housekeeping gene *β-tubulin*, used as reference gene and set to 1,000. The relative expression levels are presented as means from three independent experiments and the error bars correspond to standard errors of the means.

Figure. 3. Spatial expression profile of *Paracentrotus lividus wnt2* established by whole-mount *in situ* hybridization assays. Developmental stages are as follows: A, B: late blastula; C: swimming blastula; D, E: mesenchyme blastula; F, G: early gastrula; H: gastrula; I, J: Prism. All embryos are in lateral views with the vegetal pole toward the bottom and the oral side toward the left, unless indicated as “vv” for vegetal views with the oral side on the left.

SUPPLEMENTARY MATERIAL LEGEND

Supplementary Figure 1. Unrooted Maximum Likelihood tree used to establish the orthology of *Paracentrotus lividus* WNT2. Accession numbers of all the protein sequences used in this analysis are available in Supplementary Table 1. Bootstrap support values are indicated to the right of the nodes. Bilaterians WNT subfamilies and their support values are accordingly color-coded. *P. lividus* WNT proteins are in red and *P. lividus* WNT2 is further pinpointed by a red arrow. Species abbreviations are as follows: Dp, *Daphnia pulex* (water flea, crustacean); Hs, *Homo sapiens* (human, vertebrate); Lg, *Lottia gigantea* (limpet, mollusk); Pl, *Paracentrotus lividus* (sea urchin, echinoderm); Xt, *Xenopus tropicalis* (frog, vertebrate). Branch-lengths correspond to the number of substitution per site.

Supplementary Figure 2. Unrooted Bayesian Inference tree used to establish the orthology of *Paracentrotus lividus* WNT2. Accession numbers of all the protein sequences used in this analysis are available in Supplementary Table 1. Posterior probabilities support values are indicated to the right of the nodes. Bilaterians WNT subfamilies and their support values are accordingly color-coded. *P. lividus* WNT proteins are in red and *P. lividus* WNT2 is further pinpointed by a red arrow. Species abbreviations are as follows: Dp, *Daphnia pulex* (water flea, crustacean); Hs, *Homo sapiens* (human, vertebrate); Lg, *Lottia gigantea* (limpet, mollusk); Pl, *Paracentrotus lividus* (sea urchin, echinoderm); Xt, *Xenopus tropicalis* (frog, vertebrate). Branch-lengths correspond to the number of substitution per site.

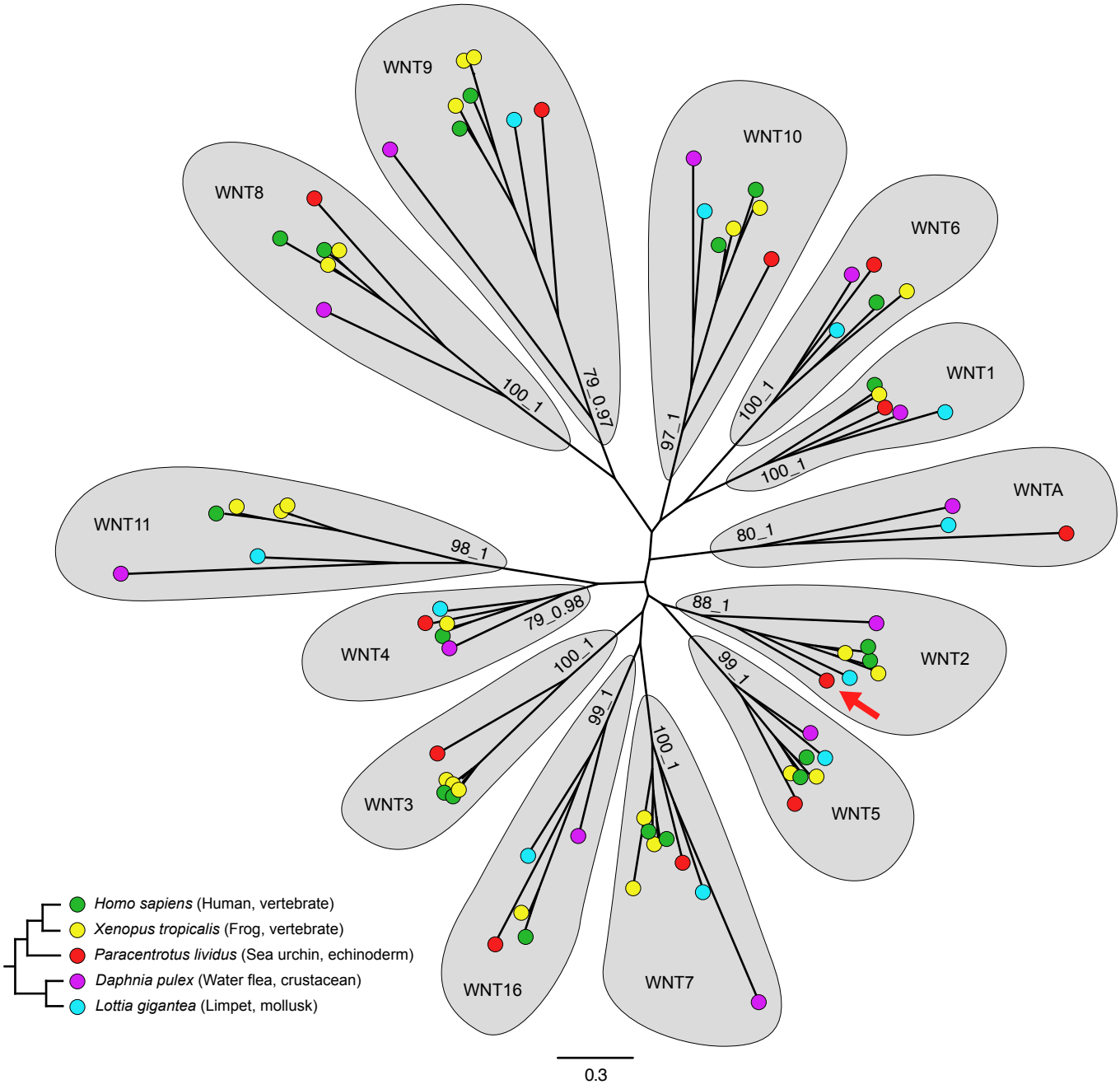
Supplementary Figure 3. Unrooted Maximum Likelihood tree used to establish the orthology of *Strongylocentrotus purpuratus* WNT2-like protein (GenBank accession number: AAA30084). Accession numbers of all other protein sequences used in this analysis are available in Supplementary Table 1. Bootstrap support values are indicated to the right of the nodes. Bilaterians WNT subfamilies and their support values are accordingly color-coded. *S. purpuratus* WNT2 is highlighted in red and further pinpointed by a red arrow. Species abbreviations are as follows: Dp, *Daphnia pulex* (water flea, crustacean); Hs, *Homo sapiens* (human, vertebrate); Lg, *Lottia gigantea* (limpet, mollusk); Pl, *Paracentrotus lividus* (sea urchin, echinoderm); Sp,

Strongylocentrotus purpuratus (sea urchin, echinoderm); Xt, *Xenopus tropicalis* (frog, vertebrate). Branch-lengths correspond to the number of substitution per site.

Supplementary Figure 4. Unrooted Bayesian Inference tree used to establish the orthology of *Strongylocentrotus purpuratus* WNT2-like protein (GenBank accession number: AAA30084). Accession numbers of all other protein sequences used in this analysis are available in Supplementary Table 1. Posterior probabilities support values are indicated to the right of the nodes. Bilaterians WNT subfamilies and their support values are accordingly color-coded. *S. purpuratus* WNT2 is highlighted in red and further pinpointed by a red arrow. Species abbreviations are as follows: Dp, *Daphnia pulex* (water flea, crustacean); Hs, *Homo sapiens* (human, vertebrate); Lg, *Lottia gigantea* (limpet, mollusk); Pl, *Paracentrotus lividus* (sea urchin, echinoderm); Sp, *Strongylocentrotus purpuratus* (sea urchin, echinoderm); Xt, *Xenopus tropicalis* (frog, vertebrate). Branch-lengths correspond to the number of substitution per site.

Supplementary Figure 5. Comparative gene synteny analysis between the *Paracentrotus lividus wnt2* locus (*P. lividus* genome scaffold 2100) and the *S. purpuratus* genome. (A) Schematic representation of the *P. lividus wnt2* locus. The ten genes located upstream and the ten genes located downstream of *P. lividus wnt2* and presenting an orthology with a *Strongylocentrotus purpuratus* sequence were selected and are represented. (B) Correspondence between *P. lividus wnt2* neighbor genes and *S. purpuratus* predicted proteins (first and second columns) and genomic position of *S. purpuratus* genes onto *S. purpuratus* scaffolds (third column). The results are shown for the ten genes upstream and the ten genes downstream of *P. lividus wnt2*. (C) Schematic representation of the *S. purpuratus* genome scaffold 1005. The scaffold includes a total of three Glean-predicted genes, two of which are orthologous to two genes located downstream of *P. lividus wnt2*. The scaffold also includes a DNA sequence that can be translated into a stretch of twelve amino acids (QTRHQCKCKHHW) encoding a possible WNT-like peptide. (D) Alignment of the possible WNT-like peptide with the eleven *S. purpuratus* WNT proteins showing that the peptide exhibits a WNT-like signature. The peptide is located in the C-terminal end of the proteins. Conserved sites are marked by dots. In (A, B) *P. lividus wnt2* is in red. In (A-C) the same color code has been used in relationship to the *S. purpuratus* scaffolds.

Supplementary Table 1. Accession numbers of all the WNT sequences used in this study. For *Homo sapiens*, *Xenopus tropicalis*, and *Paracentrotus lividus*, the accession numbers correspond to NCBI identifiers (http://www.ncbi.nlm.nih.gov/genbank). For *Daphnia pulex* and *Lottia gigantea*, the accession numbers correspond to JGI identifiers (http://genome.jgipsf.org/Lotgi1/Lotgi1.home.html). Although some of the accession numbers correspond to nucleotide sequences, note that all analyses were carried out with protein sequences.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Xenopus tropicalis (Frog, vertebrate)
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Lottia gigantea (Limpet, mollusk)
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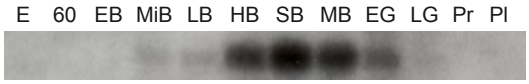
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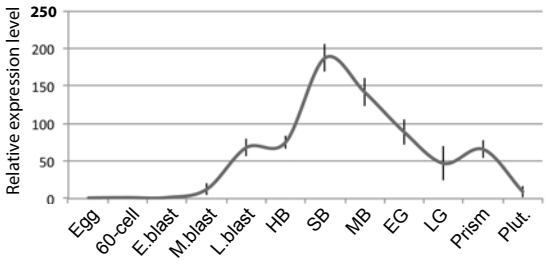
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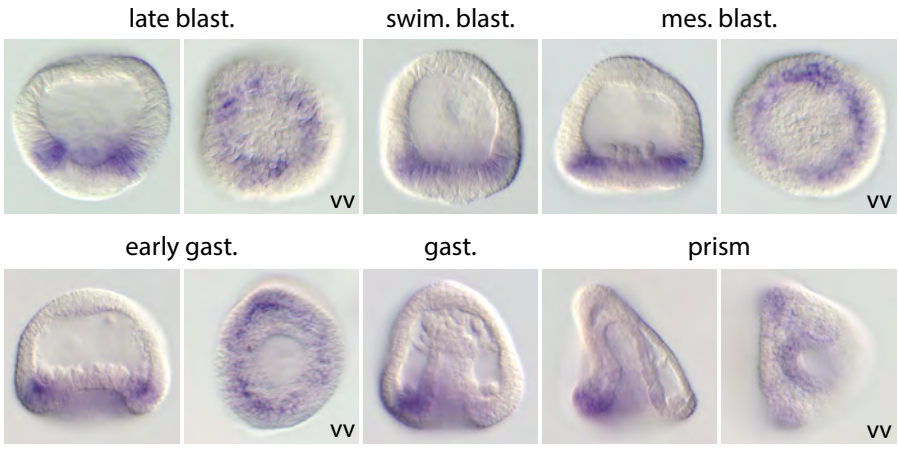
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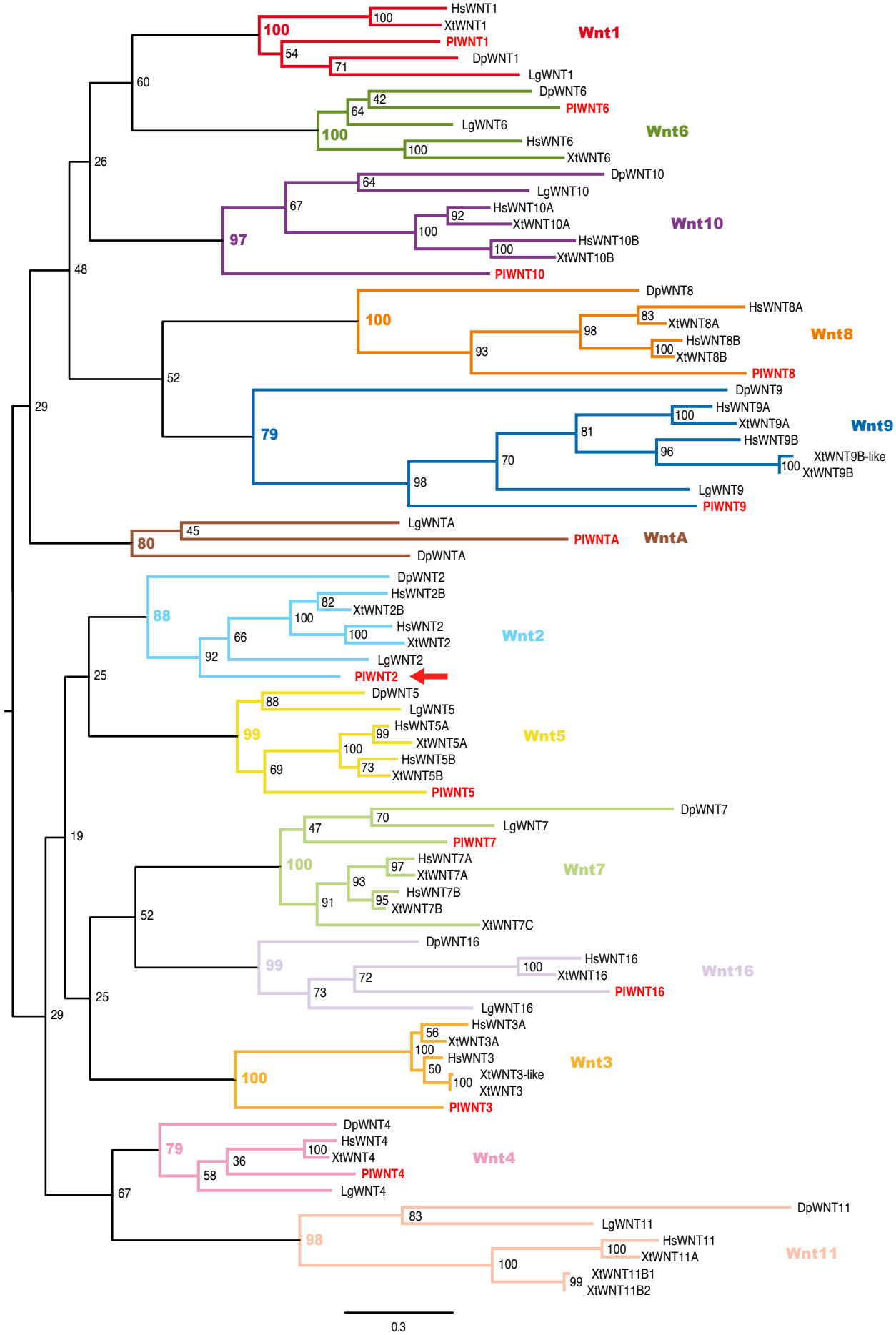
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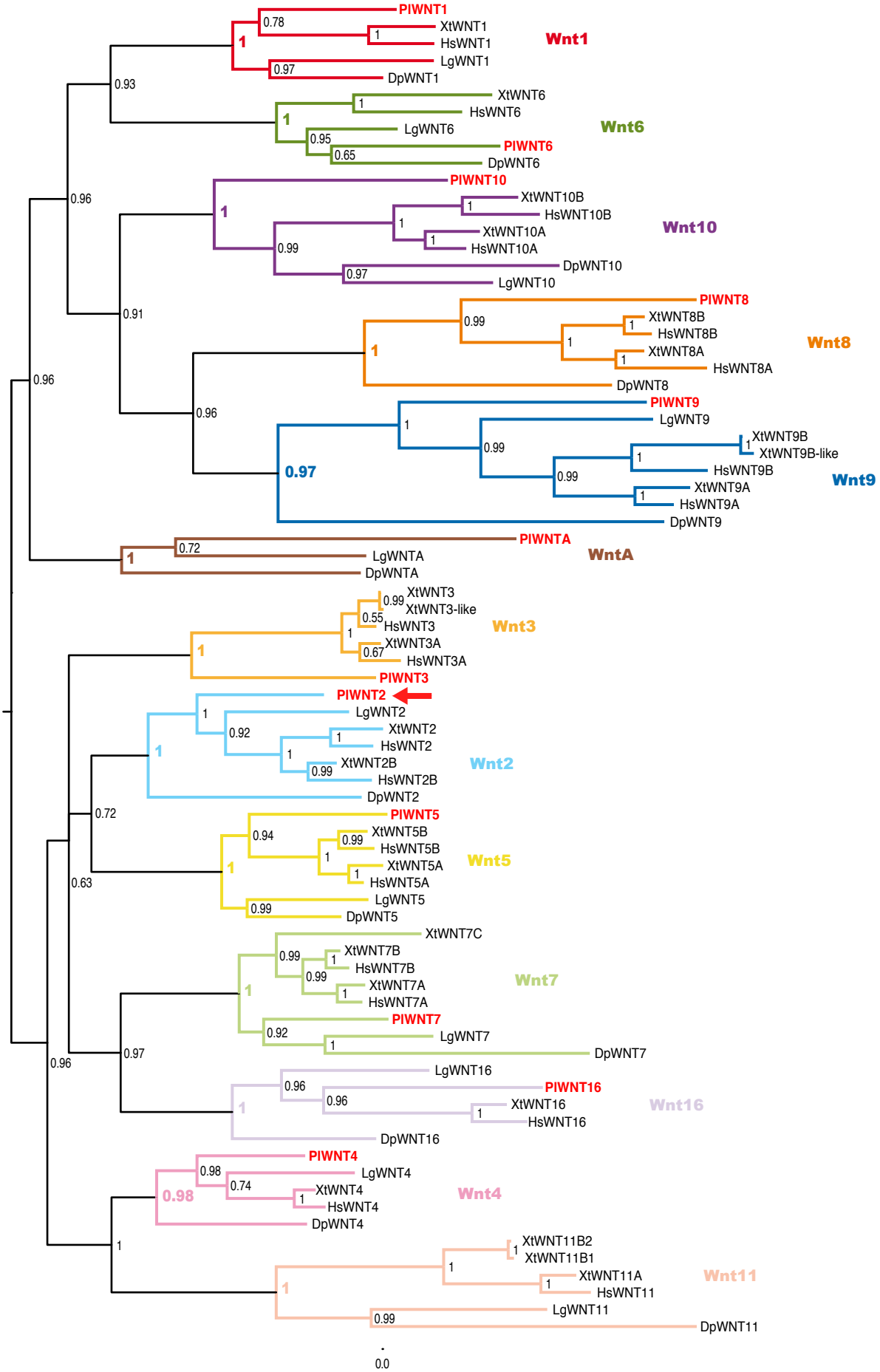
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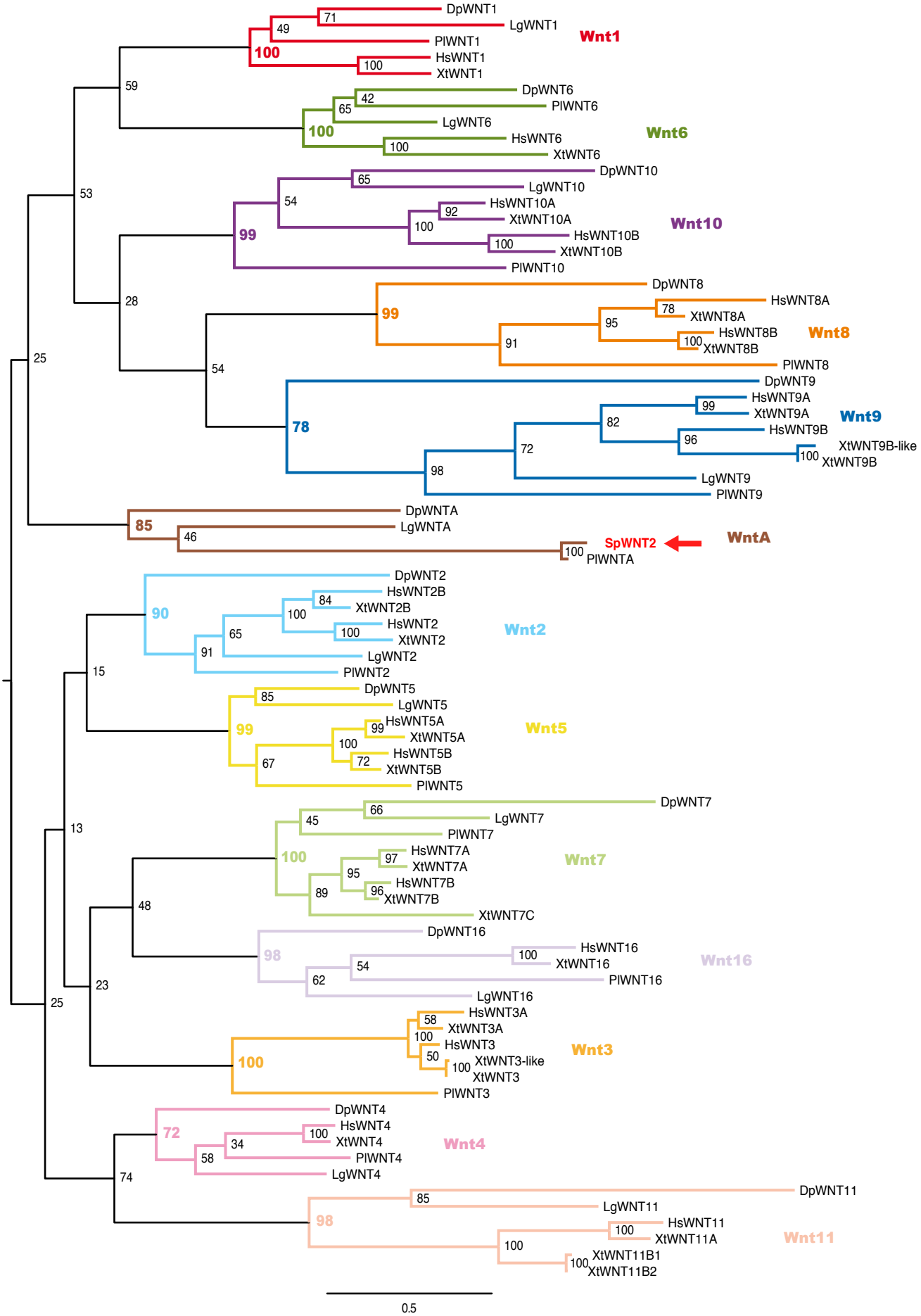
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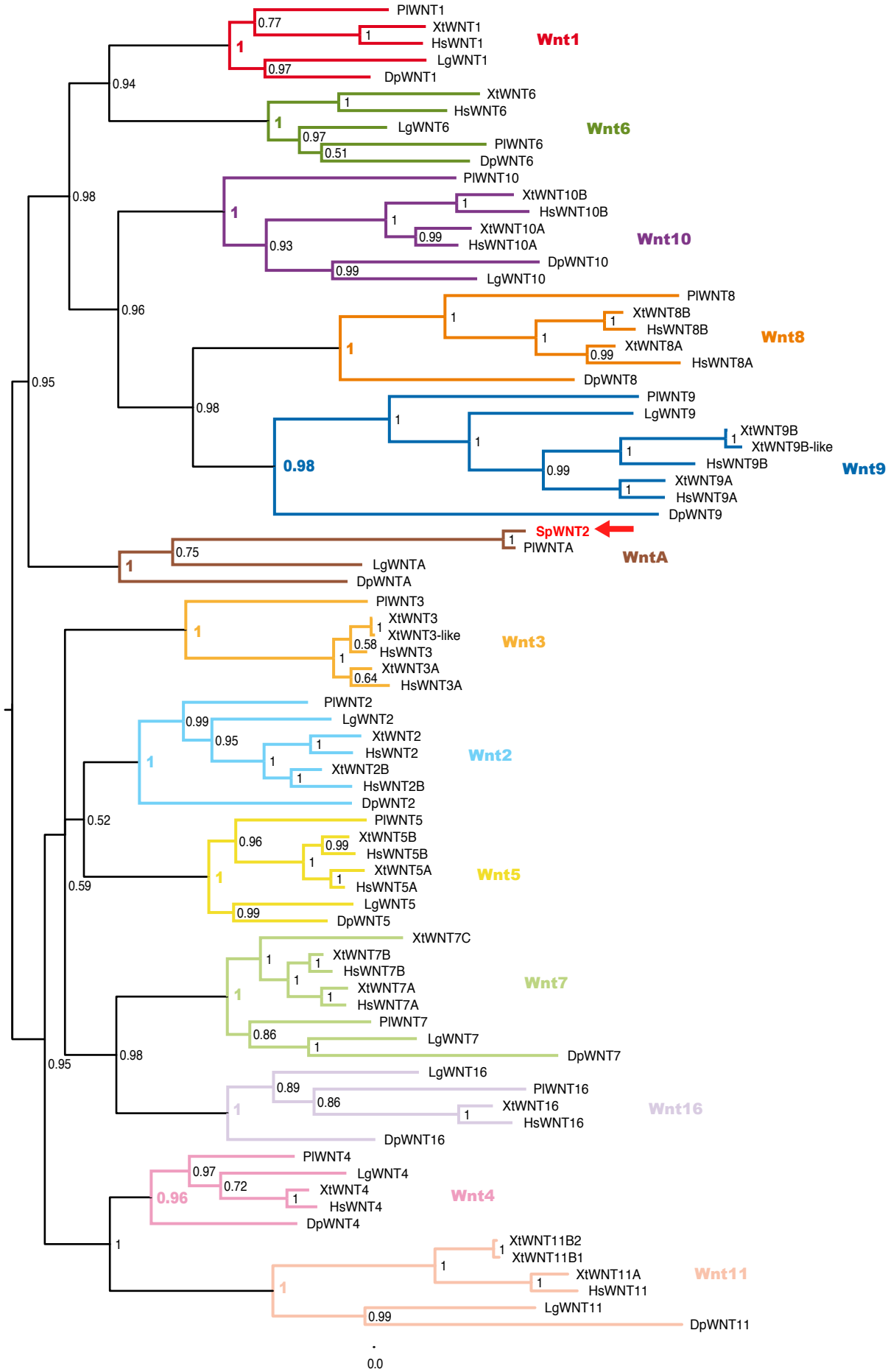
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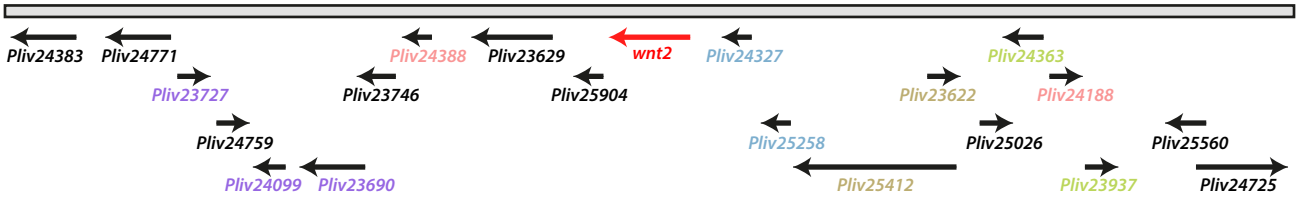

B

Upstream				Downstream		
***P. lividus* gene**	**Best BLAST hit *S. purpuratus***	**SPU_Scaffold**		***P. lividus* gene**	**Best BLAST hit *S. purpuratus***	**SPU_Scaffold**
Pliv24383.1	SPU_021093	Scaffold54		Pliv24327.1	SPU_011768	Scaffold437
Pliv24771.1	SPU_012961	Scaffold374		Pliv25258.1	SPU_011767	Scaffold437
Pliv23727.1	SPU_024104	Scaffold896		Pliv25412.1	SPU_030232	Scaffold1005
Pliv24759.1	SPU_005870	Scaffold1642		Pliv23622.1	SPU_020748	Scaffold1005
Pliv24099.1	SPU_027335	Scaffold896	***P. lividus wnt2* Pliv25201.1**	Pliv25026.1	SPU_013640	Scaffold286
Pliv23690.1	SPU_027340	Scaffold896		Pliv24363.1	SPU_023924	Scaffold53
Pliv23746.1	SPU_014572	Scaffold19		Pliv24188.1	SPU_023926	Scaffold152
Pliv24388.1	SPU_008619	Scaffold152		Pliv23937.1	SPU_003389	Scaffold53
Pliv23629.1	SPU_018532	Scaffold2307		Pliv25560.1	SPU_024462	Scaffold1208
Pliv25904.1	SPU_000536	Scaffold1257		Pliv24725.1	SPU_015088	Scaffold40

C

S. purpuratus Scaffold_1005 (226,683 bp)

D

```
                    10
peptide       QTRHQCKCKHHW
SpWNT1        KVKER.S.TF..
SpWNT3        VR.ER.D.VFV.
SpWNT4        EVIER.S..F..
SpWNT5        EVVER....FK.
SpWNT6        EK.EN.R.RF..
SpWNT7        TKIW..N..FY.
SpWNT8        VITSS.N.NFV.
SpWNT9        MVTRA.Q.RF..
SpWNT10       RRTEW.N.TF..
SpWNT16       RRVER.D..FI.
SpWNTA        HVTES.H.RFQ.
```

Animals	***Homo sapiens***	***Xenopus tropicalis***	***Paracentrotus lividus***	***Daphnia pulex***	***Lottia gigantea***
Phylum	Vertebrata		Echinodermata	Arthropoda	Mollusca
Class	Mammalia	Amphibia	Echinoidea	Branchiopoda	Gastropoda
Wnt proteins	**ACCESSION NUMBERS**				
	Protein IDs / NCBI	*Protein IDs / NCBI*	*Transcript IDs / NCBI*	*Protein IDs / JGI (all models)*	*Protein IDs / JGI*
WNT1	NP_005421.1	XP_002935152.1	KJ000369	313206	170942
WNT2	NP_003382.1	XP_012815404.1	MH427202	44143	139906
WNT2B	NP_078613.1	XP_004910783.1			
WNT3	NP_110380.1	NP_001096552.1	KJ000370		
WNT3-like		XP_002938545.1			
WNT3A	NP_149122.1	XP_002939354.2			
WNT4	NP_110388.2	NP_001239015.1	KJ000371	326278	130946
WNT5A	NP_003383.2	XP_004914236.1	HM449806	332166	130786
WNT5B	NP_110402.2	NP_001006767.1			
WNT6	NP_006513.1	XP_012826068.1	HQ322504	45092	136505
WNT7A	NP_004616.2	XP_012817241.1	KJ000372	332165	179503
WNT7B	NP_478679.1	NP_001120105.1			
WNT7C		XP_017951846.1			
WNT8A	NP_490645.1	NP_001017208.1	HM449816	315869	
WNT8B	NP_003384.2	XP_004915998.1			
WNT9A	NP_003386.1	XP_017950230.1	KJ000373	44830	180028
WNT9B	NP_003387.1	NP_001096553.1			
WNT9B-like		XP_002945358.3			
WNT10A	NP_079492.2	XP_002934004.2	KJ000374	313204	136550
WNT10B	NP_003385.2	NP_001072771.1			
WNT11A	NP_004617.2	NP_001121530.1		321275	109316
WNT11B1		NP_001008133.1			
WNT11B2		NP_001016735.1			
WNT16	NP_476509.1	NP_001096551.1	KJ000375	48424	105620
WNTA			KJ000376	328612	152125