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ORIGINAL ARTICLE

Wnt gene loss in flatworms

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Abstract *Wnt* genes encode secreted glycoproteins that act in cell–cell signalling to regulate a wide array of developmental processes, ranging from cellular differentiation to axial patterning. Discovery that canonical Wnt/β-catenin signalling is responsible for regulating head/tail specification in planarian regeneration has recently highlighted their importance in flatworm (phylum Platyhelminthes) development, but examination of their roles in the complex development of the diverse parasitic groups has yet to be conducted. Here, we characterise *Wnt* genes in the model tapeworm *Hymenolepis microstoma* and mine genomic resources of free-living and parasitic species for the presence of Wnts and downstream signalling components. We identify orthologs through a combination of BLAST and phylogenetic analyses, showing that flatworms have a highly reduced and dispersed complement that includes orthologs of only five subfamilies (Wnt1, Wnt2, Wnt4, Wnt5 and Wnt11) and fewer paralogs in parasitic flatworms (5–6) than in planarians (9). All major signalling components are identified, including antagonists and receptors, and key binding domains are intact, indicating that the canonical (Wnt/β-catenin) and non-canonical (planar cell polarity and Wnt/Ca^{2+}) pathways are functional. RNA-Seq data show expression of all *Hymenolepis* Wnts and most downstream components in adults and larvae with the notable exceptions of *wnt1*, expressed only in adults, and *wnt2* expressed only in larvae. The distribution of Wnt subfamilies in animals corroborates the idea that the last common ancestor of the Cnidaria and Bilateria possessed all contemporary Wnts and highlights the extent of gene loss in flatworms.

Keywords Wnt · β-Catenin · Platyhelminthes · Cestoda · *Hymenolepis*

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Introduction

Wnt genes encode secreted glycoproteins, typically between 350 and 400 amino acids in length, characterised by the presence of 23–25 conserved cysteine residues and by homology to the original *Drosophila* gene *wingless* (*wg*) and murine *Int1* (Cadigan and Nusse 1997). These extracellular ligands are involved in highly conserved cell–cell signal transduction pathways in metazoans that regulate fundamental processes including proliferation and differentiation, polarity, migration and apoptosis, with perturbations in Wnt signalling implicated in a wide array of degenerative diseases and cancers (see Logan and Nusse 2004). In addition, *Wnt* genes are involved in many aspects of morphogenesis and have been found to act in concert with *Hox* genes to establish anteroposterior (AP) polarity during embryogenesis (Ryan and Baxevanis 2007). Traditionally, Wnts are described as acting in three discrete pathways involving different extracellular, cytoplasmic and nuclear components: the canonical Wnt/β-catenin, non-canonical Wnt/planar cell polarity and Wnt/Ca^{2+}-dependent pathways. However, it has been recently suggested that Wnt signalling is more integrated and complex than previously thought, and it

appears that there may be a significant amount of promiscuity between ligands and their receptors, with each Wnt able to act in multiple pathways (van Amerongen and Nusse 2009).

Wnt signalling was first discovered for its role in specifying segment polarity in *Drososphila* embryogenesis (Baker 1987). Details of the transduction pathways stimulated by Wnts have since been well elucidated in major model organisms (Coudreuse and Korswagen 2007; Pires-daSilva and Sommer 2002; Wodarz and Nusse 1998), and human diseases relating to malfunctioning in Wnt signalling have received much attention (Bienz and Clevers 2000; Polakis 2000). In the last few years, characterisation of non-model animal genomes has enabled investigation of Wnt pathway components in a broader sampling of organisms, including early branching groups such as the pre-metazoan choanoflagellates (King et al. 2008) and the pre-bliaterian sponges (Adamska et al. 2007; Lapébie et al. 2009), placozoans (Srivastava et al. 2008), ctenophores (Pang et al. 2010) and cnidarians (Guder et al. 2006; Lee et al. 2006; Sullivan et al. 2007), showing that the *Wnt* gene family is a metazoan innovation (Richards and Degnan 2009).

The Lophotrochozoa is the least-studied major bilaterian clade (Giribet 2008), albeit knowledge of *Wnt* gene components and expression patterns have now been investigated in both molluscs and annelid worms (Cho et al. 2010; Prud'homme et al. 2002) and we have a comparatively good knowledge of Wnt signalling in planarian flatworms (De Robertis 2010), owing to their importance as models of regeneration (see Newmark and Sánchez Alvarado 2002; Salò 2006). Indeed, the significance of Wnt signalling in the development of flatworms (phylum Platyhelminthes) was recently underscored by the discovery that β-catenin, a downstream component of the canonical pathway, is responsible for regulating head vs. tail development during planarian regeneration (Gurley et al. 2008; Iglesias et al. 2008; Petersen and Reddien 2008; Tanaka and Weidinger 2008). It was later shown that β-catenin-independent pathways also operate during planarian regeneration to regulate neuronal organisation and growth (Adell et al. 2009), and that Wnts are involved throughout the initial wounding response (Gurley et al. 2010). These findings have helped to resolve one of the central questions of flatworm regeneration, but most basic aspects of morphogenesis in the phylum remain poorly understood, especially in the parasitic groups (Olson 2008) where research is focused primarily on medically relevant aspects of their biology (e.g. Maule et al. 2006). The first report to focus on Wnt signalling in parasitic flatworms has concerned the human bloodfluke *Schistosoma japonicum*, demonstrating that Wnt4 stimulates the canonical pathway (Li et al. 2010). Beyond this, investigation of Wnt signalling components has not been addressed in the phylum outside planarians.

Here, we characterise *Wnt* genes in a model tapeworm, *Hymenolepis microstoma*, through empirical and in silico approaches and mine complete genome assemblies of *Hymenolepis* and other flatworms to identify the presence of Wnts and downstream components of Wnt signalling. Gene identities are assigned using flatworm Wnts as BLASTP queries against the human genome and by phylogenetic analysis of lophotrochozoan Wnt sequences. We investigate viability of known Wnt pathways in *Hymenolepis* by examining the integrity of functional domains and evidence of expression in different phases of the tapeworm life cycle using RNA-Seq data. Combining this information with the reported distribution of Wnts in Metazoa, we estimate gain/loss of Wnt subfamilies through animal evolution and discuss briefly the roles of Wnt signalling in the processes of axial patterning and segmentation in the evolution of tapeworms.

Materials and methods

Genomic resources

Genomic and transcriptomic data from a laboratory strain of *H. microstoma* (Platyhelminthes: Cestoda: Cyclophyllidea) maintained in vivo using BALB/c mice and flour beetles (see Cunningham and Olson 2010, for a complete description of the model system) were used to identify orthologs of Wnts and associated signalling components (Table 1). Several *Wnt* genes were first identified from partial transcripts shot-gun characterised via Roche 454-based sequencing, and representing whole adult and larval worm cDNAs (unpublished data). These data were used to create gene-specific primers for PCR-RACE, and 3′ RACE reactions were obtained using a Clontech SMART RACE kit for three Wnts. At the same time, through collaboration with the Wellcome Trust Sanger Institute, a combination of Roche 454 and Illumina Solexa next generation sequencing technologies were used to characterise (>40× coverage) and assemble the 147 Mb genome of *H. microstoma* (see Olson et al. 2011, publically available from http://www.sanger.ac.uk/resources/downloads/helminths/hymenolepis-microstoma.html). In addition, non-normalised cDNA samples of expressed genes representing whole adult worms and 5-day-old (i.e. mid-metamorphosis) larvae were characterised by Illumina Solexa paired-end next generation sequencing (NGS) and mapped to the genome via RNA-Seq (Wang et al. 2009) to aid in the annotation of coding regions of the genome. Assembled genomic and transcriptomic data were queried and analysed using Geneious v. 5.3.6 (Drummond et al. 2010), and Artemis (Carver et al. 2008; Rutherford et al. 2000) development v. 13 was used to view and quantify RNA-Seq data. Progress in genome characterisation through the course of the study eventually

Table 1 Wnt signalling components in parasitic and free-living flatworms

Wnt pathway/ role Component gene	*Hymenolepis microstoma* (mouse bile-duct tapeworm)	*Echinococcus multilocularis* (hydatid tapeworm)	*Echinococcus granulosus* (hydatid tapeworm)	*Schistosoma mansoni* (human blood fluke)	*Schmidtea mediterranea* (planarian)
Wnt/β-catenin					
Wnt	6	6	6	5	9
Frizzled	8	7	8	9	13
Dishevelled	✓	✓	✓	✓	✓
GSK3	✓	✓	✓	✓	✓
APC	✓	✓	✓	✓	✓
Axin	✓	✓	✓	✓	✓
β-catenin	✓	✓	✓	✓	✓
LEF/TCF	✓	✓	✓	✓	✓
Ca^{2+} dependent					
Phospholipase C	✓	✓	✓	✓	✓
CaMKII	✓	✓	✓	✓	✓
Planar cell polarity					
Rho GTPase	✓	✓	✓	✓	✓
JNK	✓	✓	✓	✓	✓
Antagonists					
Dickkopf	0	0	0	0	0
WIF	✓	✓	✓	✓	✓
Cerberus	0	0	0	0	0
SFRP	✓	✓	✓	✓	✓

Numbers of Wnt and Frizzled orthologs are shown for each species, whereas checkmarks indicate the presence of one or more orthologs
GSK3 glycogen synthase kinase 3, *APC* adenomatous polyposis coli, WIF Wnt inhibitory factor, *SFRP* secreted frizzled related proteins

enabled transcript sequences to be determined in silico using RNA-Seq coverage to identify ORFs and related exons. In silico analyses corroborated the transcript sequences previously characterised by RACE and superseded the need for further such characterisations.

Hymenolepis orthologs were used as BLAST queries to identify orthologs in available parasitic flatworm genomes, which included the tapeworms *Echinococcus multilocularis* and *Echinococcus granulosus* and the human bloodfluke *Schistosoma mansoni* (all available from http://www.sanger.ac.uk/resources/downloads/helminths/). In addition, we mined genome data of the planarian model *Schmidtea mediterranea* (http://smedgd.neuro.utah.edu/) and of the marine flatworm *Macrostomum lignano* (http://www.mac genome.org/genome.html). Evaluation of the *Macrostomum* data, however, proved the assembly to be too preliminary to produce reliable estimates of gene content and the species was thus excluded. We also used published gene sequences and mined genomic resources for additional lophotochozoan taxa including a leech (*Helobdella*, http://genome.jgi-psf.org/Helro1/Helro1.home.html), mollusc (*Lottia*, http://genome.jgi-psf.org/Lotgi1/Lotgi1.home.html) and annelid worm (*Capitella*, http://genome.jgi-psf.org/Capca1/Capca1.home.html).

Identification of Wnt pathway components, functional domains and expression

Major downstream components (Table 1; Supplementary File 1) of the three Wnt pathways were identified in flatworm genomes using a reciprocal BLAST approach: human, *Xenopus* and *Drosophilla* orthologs were used for TBLASTX searches against the platyhelminth genome data, and identified genes in turn screened against the NCBI non-redundant nucleotide database (nr). Integrity of the genes was examined by identifying signal peptides, binding and other functional domains with the aid of the Conserved Domain Database of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and SignalP (http://www.cbs.dtu.dk/services/SignalP/). Uninterrupted ORFs and characteristic motifs in such functional domains were considered evidence of gene conservation and thus functional viability (Richards and Degnan 2009). We also examined evidence of expression in *Hymenolepis* for each of the Wnts and associated elements during both adult and larval phases of the life cycle by examining RNA-Seq data. As currently available data represent single estimates of the adult and larval transcriptomes, we could not apply

statistics to the differences in expression levels. Instead the number of reads per kilobase of transcript per million mapped reads (i.e. RPKM; Mortazavi et al. 2008) was calculated and used as a measure of gene expression independently in both adults and larvae. This method takes into account the influence of molar concentration and transcript length and thus provides an objective comparison of transcript level among genes in each sample. RPKM values were calculated using Artemis.

Phylogenetic analyses

Amino acid sequences of flatworm and other lophotrochozoan Wnts (see Fig. 1 and Supplementary File 1 for sequence accession numbers) were aligned across the highly conserved cysteine-rich binding regions. Positions with alignment gaps in a majority of the sequences were removed and the regions concatenated, resulting in an alignment (available from www.olsonlab.com) of 191 residues shown here as a 50% consensus with invariant sites underlined and concatenated regions separated by dashes: 5′ XXRACSXGXLXX CXCDXXXXXXXXXXXWXWGGCSDNXXFGXXFXRX F X D X X E X X X X – X L M N L H N N X A G R X X V X XXXXXXCKCHGVSGSCXXKTCWXXLPFRXXGXX LKXXYXXAXXVXX–XXXXXXDLVYXXXSPBYCX– X B X X G X B X C X X X C C G R G Y X T X X X X X X E XCXCKFXWCCXVXCXXCXXXXXXXXCX 3′. Phylogenetic relationships of the genes were estimated via Bayesian inference (Ronquist and Huelsenbeck 2003) using the WAG (Whelan and Goldman 2001) amino acid substitution model including gamma among-site rate variation. Analyses were run for 1,100,000 generations, with samples saved every 200 generations, and the first 50,000 generations eliminated as 'burn-in' prior to forming a consensus tree using the *sumt* command.

Presence/absence of metazoan Wnt genes reported here and in the literature were tabulated and scored as binary characters using MacClade (Maddison et al. 2005). The distribution of states was then mapped to the metazoan phylogeny of Hejnol et al. (2009), with further resolution of arthropd interrelationships based on Telford et al. (2008) and Regier et al. (2010). Because orthologous genes share a most recent common ancestor by definition, gain/loss was modelled using Dollo parsimony, which allows a single gain and multiple, independent losses.

Results and discussion

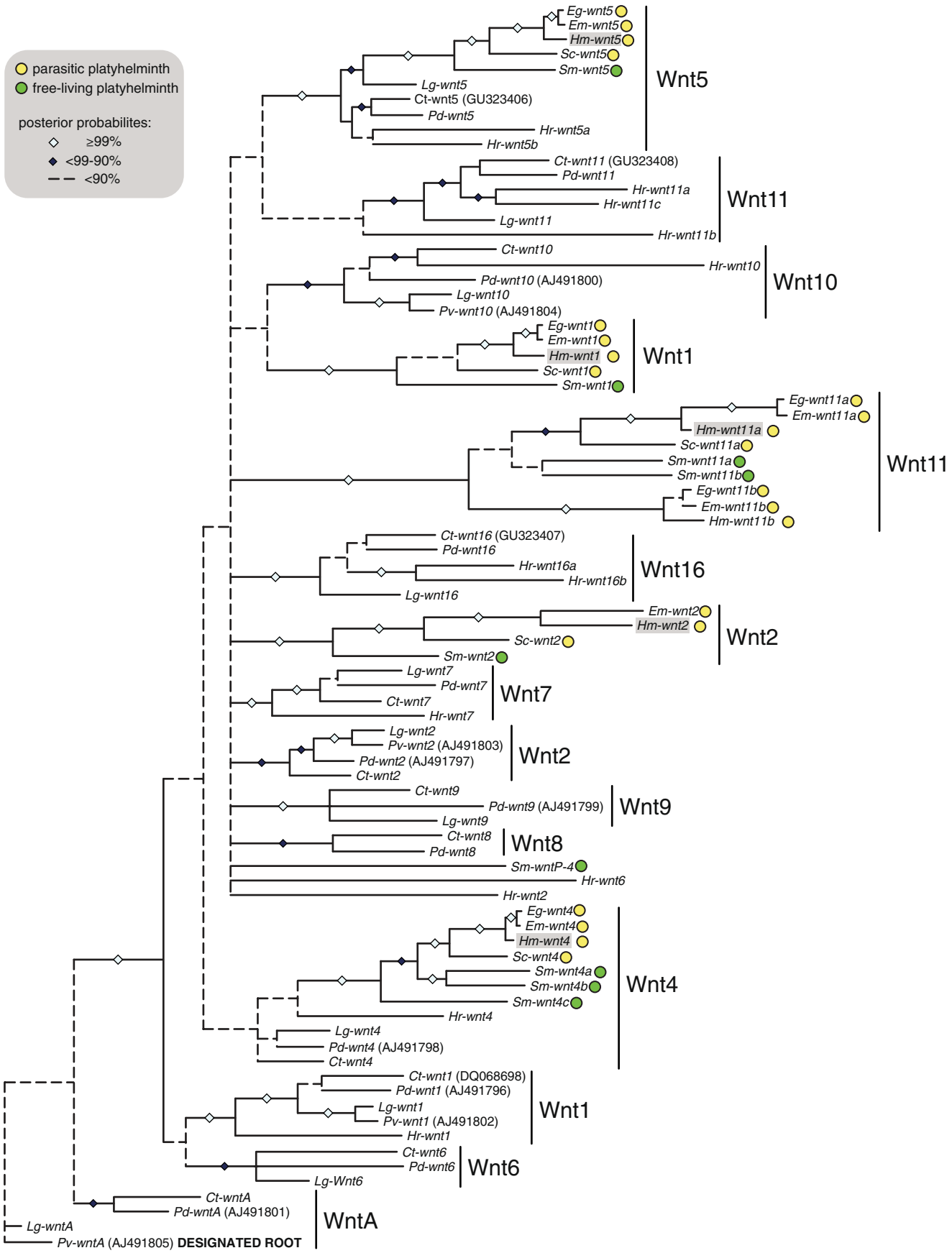
Wnt genes in flatworms

Empirical and in silico approaches show that the *H. microstoma* genome contains a total of six *Wnt* genes, representing five subfamilies: *Hm-wnt1*, *wnt2*, *wnt4*, *wnt5* and *wnt11a/b*. Identification of Wnts in other flatworms shows the presence of the same subfamilies, with single orthologs of *wnt1*, *wnt2* and *wnt5* and two paralogs of *wnt11* in all flatworms except the bloodfluke *S. mansoni*, which lacks *wnt11b*. In addition, we find that the parasitic flatworms possess a single ortholog of *wnt4*, whereas the planarian *S. mediterranea* possesses three paralogs. Full-length transcripts, where deduced, are typical in structure and length and contain a signal peptide, a number of *N*-glycosylation sites and highly conserved cysteine residues that ensure their proper folding and secretion (Nusse and Varmus 1992).

Identification of the genes on the basis of phylogenetics alone (Fig. 1) was not possible as flatworm orthologs are highly divergent and grouped with other lophotrochozoan orthologs only in the cases of the *Wnt4* and *Wnt5* subfamilies, and only with strong support in *Wnt5* (Fig. 1). For all subfamilies present in flatworms, however, orthologs grouped together with high support and showed monophyly of the parasitic species relative to *Schmidtea*, as expected based on our understanding of flatworm interrelationships (Olson and Tkach 2005). Moreover, despite divergence in flatworm Wnts, BLAST analysis resulted in consistent hits to known Wnt subfamilies, and thus a combination of BLAST and phylogenetics was effective in classifying the genes. Recently, *Wnt* genes were identified and classified in *S. mediterranea* by Gurley et al. (2008), but their analysis grouped six of the nine genes into a single clade, weakly supported as the sister group to a clade of other metazoan *Wnt11* orthologs. By restricting our analyses to lophotrochozoans and including additional flatworm Wnts, we were able to reclassify several of the *Smed-′Wnt11′* genes as paralogs of the *Wnt4* subfamily (i.e. *Sm-wnt4a*, was *wnt11-6*; *Sm-wnt4b*, was *wnt11-5*; *Sm-wnt4c*, was *wnt11-3*), whereas *wntP-4* (i.e. Smed-wnt11-3; Gurley et al. 2010) failed to group with any other sequence and could not be verified as an ortholog of *Wnt11*.

No two *Wnt* genes were found on the same contig in the flatworm genome assemblies despite contig/scaffold lengths in excess of 100 Kb in many cases, and over 10 Mb in the

Fig. 1 Bayesian inference analysis of flatworm and other lophotrochozoan Wnt genes based on a concatenated alignment of 191 amino acid residues encompassing the conserved cysteine-rich regions. Nodal support shown as posterior probabilities where ≥90%; support below 90% shown as *dashed lines*. Contig/scaffold IDs containing flatworm Wnts are found in Supplementary File 1, whereas those of other lophotrochozoans are found in Cho et al. (2010) and Janssen et al. (2010), or the accession numbers are shown parenthetically. *Abbreviations: Ct Capitella teleta* (polychaete), *Eg Echinococcus granulosus* (dog/fox tapeworm), *Em Echinococcus multilocularis* (fox tapeworm), *Hm Hymenolepis microstoma* (mouse tapeworm), *Hr Helobdella robusta* (leech), *Lg Lottia gigantea* (mollusc), *Pd Platynereis dumerilii* (polychaete), *Pv Patella vulgata* (limpet), *Sc Schistosoma mansoni* (bloodfluke), *Sm Schmidtea mediterranea* (planarian) ▶


parasitic platyhelminth
free-living platyhelminth
posterior probabilites:
≥99%
<99-90%
<90%
Eg-wnt5
Em-wnt5
Hm-wnt5
Sc-wnt5
Sm-wnt5
Lg-wnt5
Ct-wnt5 (GU323406)
Pd-wnt5
Hr-wnt5a
Hr-wnt5b
Wnt5
Ct-wnt11 (GU323408)
Pd-wnt11
Hr-wnt11a
Hr-wnt11c
Lg-wnt11
Hr-wnt11b
Wnt11
Ct-wnt10
Hr-wnt10
Pd-wnt10 (AJ491800)
Lg-wnt10
Pv-wnt10 (AJ491804)
Wnt10
Eg-wnt1
Em-wnt1
Hm-wnt1
Sc-wnt1
Sm-wnt1
Wnt1
Eg-wnt11a
Em-wnt11a
Hm-wnt11a
Sc-wnt11a
Sm-wnt11a
Sm-wnt11b
Eg-wnt11b
Em-wnt11b
Hm-wnt11b
Wnt11
Ct-wnt16 (GU323407)
Pd-wnt16
Hr-wnt16a
Hr-wnt16b
Lg-wnt16
Wnt16
Em-wnt2
Hm-wnt2
Sc-wnt2
Sm-wnt2
Wnt2
Lg-wnt7
Pd-wnt7
Ct-wnt7
Hr-wnt7
Wnt7
Lg-wnt2
Pv-wnt2 (AJ491803)
Pd-wnt2 (AJ491797)
Ct-wnt2
Wnt2
Ct-wnt9
Pd-wnt9 (AJ491799)
Lg-wnt9
Wnt9
Ct-wnt8
Pd-wnt8
Wnt8
Sm-wntP-4
Hr-wnt6
Hr-wnt2
Eg-wnt4
Em-wnt4
Hm-wnt4
Sc-wnt4
Sm-wnt4a
Sm-wnt4b
Sm-wnt4c
Hr-wnt4
Lg-wnt4
Pd-wnt4 (AJ491798)
Ct-wnt4
Wnt4
Ct-wnt1 (DQ068698)
Pd-wnt1 (AJ491796)
Lg-wnt1
Pv-wnt1 (AJ491802)
Hr-wnt1
Wnt1
Ct-wnt6
Pd-wnt6
Lg-Wnt6
Wnt6
Ct-wntA
Pd-wntA (AJ491801)
Lg-wntA
Pv-wntA (AJ491805) DESIGNATED ROOT
WntA

reference genome of the fox tapeworm *E. multilocularis*. Moreover, in *Hymenolepis* we verified that *Wnt* gene regions are flanked by non-*Wnt* genes, and thus we find no evidence of the *Wnt* gene synteny reported in other metazoans (Cho et al. 2010; Sullivan et al. 2007). Thus, as is the case for *Hox* genes (Koziol et al. 2009; Olson et al. 2011; Olson 2008), flatworms have lost the ancestral clustered organisation typical of these gene families.

Wnt pathway components in flatworms

As well as mining flatworm genomes for Wnt ligands, we searched extensively for associated downstream components of Wnt signalling, finding that flatworms possess a full complement of genes associated with each of the three known pathways (Table 1), save the antagonists Dickkopf (DKK) and Cerberus (CER). We also investigated the integrity of conserved binding domains in putative *Hymenolepis* orthologs of β-catenin (Bcat), frizzled (Frz), dishevelled (Dsh), adenomatous polyposis coli (APC) and T-cell-specific transcription factor/lymphoid enhancer binding factor (TCF/LEF) (Fig. 2). Both *Hm-fzdB* and *Hm-dsh* contain all the binding sites characteristic of active signalling, including the Wnt binding cysteine-rich domain and seven transmembrane domains in Frz, and all three key domains found in metazoan Dsh (i.e. DIX, PDZ and DEP). *Hm-frzB* also contains a signal peptide and the intracellular KTXXXW (KTLVSW) that binds the PDZ domain of Dsh, and is essential for Wnt/β-catenin-dependent signalling (Umbhauer et al. 2000). Further downstream, *Hm-Bcat* contains the 12 armadillo repeats that bind TCF/LEF and proteins involved in the destruction complex (i.e. APC and Axin); however, as we were unable to deduce full-length transcripts in some cases, we could not locate the upstream glycogen synthase kinase 3 (GSK3) phosphorylation domain that ensures its degradation via ubiquitination. Also, although *Hm-TCF/LEF* contains the Sox-Tcf high mobility group domain required to bind DNA, we were unable to find the β-catenin binding domain. Nevertheless, the presence of characteristic and intact binding sites is strongly indicative of the canonical pathway (and potentially non-canonical pathways) being under functional selective pressure in flatworms, and thus that Wnt/β-catenin-dependent signalling is active throughout the phylum. Moreover, although flatworm orthologs are divergent, their otherwise typical gene structures suggest they function in bilaterian-canonical roles, and not in lineage-specific ways as inferred from the less characteristic gene structures found in pre-bilaterian groups (Richards and Degnan 2009), or from the presence of large numbers of independent paralogs as seen in vertebrates (Garriock et al. 2007).

Expression of canonical Wnt pathway components in *Hymenolepis*

Table 2 shows expression levels of canonical Wnt pathway components in the model tapeworm *H. microstoma*. It should be noted that these values are based on single cDNA pools shot-gun sequenced via NGS, and that, as the adult and larval concentrations were not normalised to each other, direct comparison cannot be made between developmental stages. Nevertheless, these data provide the first snapshot of Wnt expression in the life cycle of a tapeworm and allow us to predict which components are most likely to be functionally active in adult and larval development. Although all components had at least some RNA-Seq hits, we considered expression levels less than an RPKM value of 1 to indicate an effective lack of expression, which included *Hm-wnt1* and *Hm-frzD* in larvae and *Hm-wnt2* and *Hm-TCF/LEF* in adults. Also noteworthy is the low level of expression of *Hm-wnt2* in larvae, raising the possibility that this gene is not expressed in either developmental stage, and *Hm-wnt4*, that shows high levels of expression in adults, and >10-fold higher expression than other Wnts in larvae. Other components show varying levels in both phases of the life cycle, with Frz receptors exhibiting particularly high expression levels and diversity.

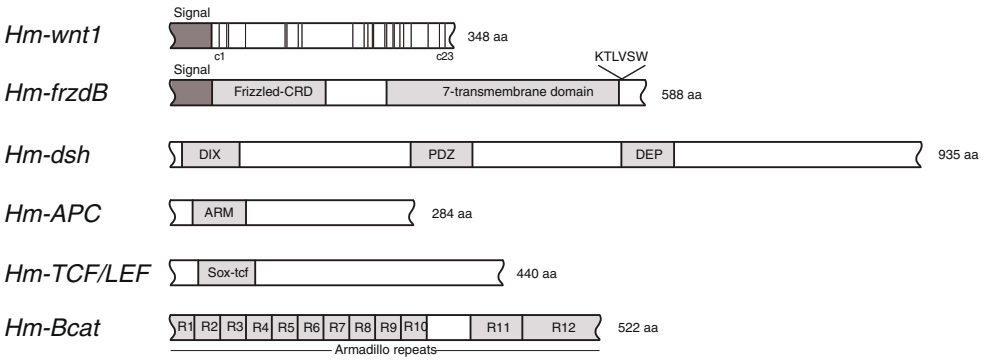


Fig. 2 Functional domains of Wnt pathway components in *H. microstoma* orthologs

Table 2 RPKM values indicating RNA-Seq expression levels of Wnts and canonical Wnt pathway components during adult and larval development in *Hymenolepis microstoma*

Gene	RPKM[a]	
	Adult	Larval
Hm-wnt1	3.7	0.2
Hm-wnt2	0.6	1.7
Hm-wnt4	6.1	167.4
Hm-wnt5	5.1	10.3
Hm-wnt11a	1.2	10.6
Hm-wnt11b	4.5	13
Hm-frzA	4.2	94.1
Hm-frzB	4.9	72.7
Hm-frzC	6.6	10.1
Hm-frzD	1.9	0.5
Hm-frzE	3.5	96.3
Hm-frzF	11.4	14.9
Hm-frzG	12.6	47.3
Hm-frzH	3.4	132.4
Hm-dsh	2.3	18.5
Hm-axin	2	18.6
Hm-APC	1.5	12.8
Hm-GSK3	15	18.4
Hm-Bcat	11.5	128.3
Hm-TCF/LEF	0.7	10.3

[a] RPKM values show the number of reads mapping to the genome per kilobase of transcript per million reads sequenced, and thus corrects for sample differences in molar concentration and transcript length

Wnt loss in flatworms

Figure 3 shows gain and loss of Wnt subfamilies in Metazoa according to recent hypotheses of their interrelationships and the a priori assumption that each gene subfamily is monophyletic. The origin of Wnts is associated with the emergence of multicellularity and recognisable orthologs have not been identified in the genome of the unicelluar choanoflagellates (King et al. 2008). Pre-bilaterian lineages such as the comb jellies, sponges and placozoans possess a handful of recognisable *Wnt* genes (Pang et al. 2010), whereas an enormous expansion in ligand diversity appears to have occurred in the most recent common ancestor (MRCA) of the Cnidarian and Bilateria (Lee et al. 2006), resulting in the origin of 11 of the 13 contemporary subfamilies. Protostomes are characterised by the loss of the Wnt3 subfamily, and thus have an ancestral condition of 12 genes, albeit many instances of lineage-specific loss are seen in the evolution of both Lophotrochozoa and Ecdysozoa (Cho et al. 2010; Janssen et al. 2010). For example, molluscs are characterised by the loss of *Wnt8*, and insects by the loss of *Wnt16* (Murat et al. 2010), and significant further reductions are found in specific lineages, such as the limpet *Patella* that possesses only four Wnts (Prud'homme et al. 2002). By comparison, the evolution of flatworms appears coincident with a very large primary loss in Wnt diversity, totalling seven subfamilies (*Wnt6*, *Wnt7*, *Wnt8*, *Wnt9*, *Wnt10*, *Wnt16* and *WntA*), with further loss of *Wnt4* paralogs associated with the evolution of parasitism in the phylum. Although this reduction appears specific to flatworms based on available data, the extent to which loss may have been inherited from the common ancestor of the Platyzoa (sensu Hejnol et al. 2009) cannot be determined without both knowledge of Wnt complements in related minor groups such as gastrotrichs, rotifers and gnathostomulids, and better resolution of their interrelationships.

Deuterostomes inherited all 13 Wnt subfamilies, but early branching lineages show many independent losses (Fig. 2). Vertebrates are characterised by the loss of *WntA*, but also by the gain of a large number of Wnt paralogs and associated downstream components, consistent with whole genome duplications in the MRCA of the group (Garriock et al. 2007). Also typical in vertebrates is the presence of multiple Wnt antagonists, such as DKK, CER, Wnt inhibitory factor (WIF) and secreted frizzled related proteins (SFRP), and it is probable that the evolution of Wnt antagonists played an important role in catalysing the evolution of complexity in Wnt signalling. By comparison, there is a general paucity of Wnt antagonists in basal Metazoa (Pang et al. 2010), and although flatworms possess orthologs of WIF and SFRP, the lack of a netrin-like domain in *Hm-sfrp*, thought to be responsible for anchoring them to the extracellular matrix (Rattner et al. 1997), raises the possibility that it is not acting as an antagonist in parasitic flatworms.

Wnt signalling in AP patterning and animal segmentation

Wnt signalling is involved in a diverse range of cellular interactions throughout development, including regeneration (Broun 2005; Gurley et al. 2010), segmentation (Bolognesi et al. 2008; Miyawaki et al. 2004) and axial patterning (Ryan and Baxevanis 2007). Spatial and temporal expression patterns in pre-bilaterian groups such as sponges (Adamska et al. 2007) and cnidarians (Lee et al. 2006) are consistent with the hypothesis that Wnt genes were ancestrally involved in AP patterning and that Hox genes were subsequently co-opted into the process in the MRCA of the Cnidaria and Bilateria. In *Drosophila*, the *Wnt1* ortholog *wg* acts as a segment polarity gene to delineate parasegmental boundaries in concert with the homeobox gene *engrailed* (*eg*) (Baker 1987), and Wnts have subsequently been shown to play important roles in all

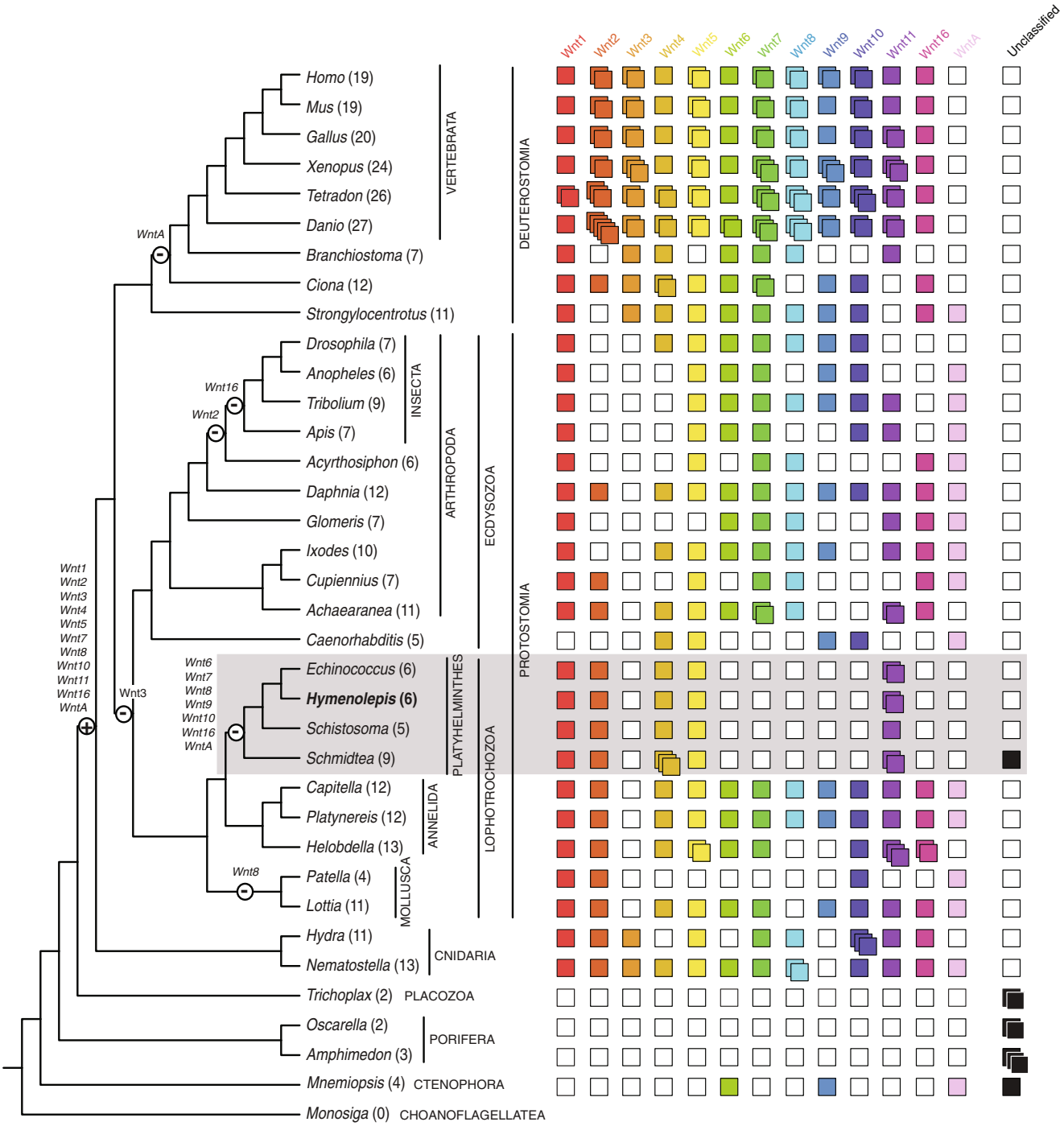


Fig. 3 Distribution of Wnt orthologs in the Metazoa showing inferred gain/loss of genes through evolution. *Filled boxes* indicate presence of putative orthologs based on analyses herein and reports from the literature, with lineage-specific paralogs shown as multiple boxes and unclassified genes shown in *black*. Metazoan phylogeny based on Hejnol et al. (2009) with the interrelationships of arthropods based on Telford et al. (2008) and Regier et al. (2010). Gain/loss of specific orthologs shown at nodes inferred on the basis of Dollo parsimony, allowing a single gain and multiple, subsequent losses. Major gains/losses of Wnts during animal evolution are illustrated at the nodes as plus/minus signs. Note major expansion of Wnts in the Cnidarian plus Bilaterian ancestor, loss of *Wnt3* in protostomes, loss of *WntA* in chordates and loss of *Wnt6*, *Wnt7*, *Wnt8*, *Wnt9*, *Wnt10 Wnt16*, and *WntA* in flatworms (*grey box*)

segmentation systems studied to date. For example, interfering with Wnt signalling during development disrupts the segmentation clock in vertebrates (Gibb et al. 2009) and produces similarly interrupted segmentation in both arachnids (McGregor et al. 2008) and insects (Bolognesi et al. 2008; Miyawaki et al. 2004). Wnts are

commonly expressed in the posterior growth zone (PGZ) (Blair 2008) which gives rise to nascent segments in chordates and annelids, and it seems likely that Wnt expression is required in the establishment and maintenance of cells forming the PGZ, possibly by preventing their differentiation (McGregor et al. 2008). That a similar *en* expression pattern is also found in the annelid *Platynereis dumerilii* suggests that this mechanism was present in the protostome ancestor (Prud'homme et al. 2003), however whether segmentation evolved once (Dray et al. 2010) or multiple times through independent co-option of modular genetic programmes (Chipman 2010) remains controversial.

Segmentation in tapeworms has been considered an anomaly by most developmental biologists (Blair 2008) and is a derived condition not only in flatworms, but in Cestoda itself, with early branching members exhibiting varying degrees of repeated body parts (as well as complete absence of segmentation) (Olson 2008; Olson et al. 2001; Olson et al. 2008). Moreover, unlike other metazoans, segmentation in tapeworms evolved as an adaptation to parasitism (by increasing fecundity), rather than for locomotion. However, the fact that there is a small number of developmental signalling systems shared by all animals (Pires-daSilva and Sommer 2002) combined with our initial insights of whole genome data of tapeworms indicating that most major gene systems are present, if reduced (Olson et al. 2011), suggests that Wnts are likely to be involved in the evolution of segmentation in Platyhelminthes as well. On the other hand, Wnt signalling is known to act on posterior growth in vertebrates and ecdysozoans (and potentially lophotrochozoans) by regulating expression of the transcription factor Caudal, which in turn activates expression of *Hox* genes (Martin and Kimelman 2009). However, as only a few ParaHox orthologs (Brooke et al. 1998) have been found among free-living platyhelminths (Salò et al. 2001) and none is found in parasitic flatworms (Koziol et al. 2009; Olson 2008), the mechanism of segmentation in tapeworms cannot involve Caudal and must therefore be modified, if not in fact distinct, from other bilaterian mechanisms for segmentation. Further investigation of Wnt expression in tapeworms, including determination of spatial expression patterns, is currently underway and will help to determine whether or not tapeworm segmentation represents a unique developmental process in animals.

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