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Phylogenetic Analysis of the *Wnt* Gene Family: Insights from Lophotrochozoan Members

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Summary

The *Wnt* gene family encodes secreted signaling molecules that control cell fate specification, proliferation, polarity, and movements during animal development [1–3]. We investigate here the evolutionary history of this large multigenic family. *Wnt* genes have been almost exclusively isolated from two of the three main subdivisions of bilaterian animals [4], the deuterostomes (which include chordates and echinoderms) and the ecdysozoans (e.g., arthropods and nematodes). However, orthology relationships between deuterostome and ecdysozoan *Wnt* genes, and, more generally, the phylogeny of the *Wnt* family, are not yet clear. We report here the isolation of several *Wnt* genes from two species, the annelid *Platynereis dumerilii* and the mollusc *Patella vulgata*, which both belong to the third large bilaterian clade, the lophotrochozoans (which constitute, together with ecdysozoans, the protostomes). Multiple phylogenetic analyses of these sequences with a large set of other *Wnt* gene sequences, in particular, the complete set of *Wnt* genes of human, nematode, and fly [2], allow us to subdivide the Wnt family into 12 subfamilies. At least nine of them were already present in the last common ancestor of all bilaterian animals, and this further highlights the genetic complexity of this ancestor. The orthology relationships we present here open new perspectives for future developmental comparisons.

Results and Discussion

Previous phylogenetic analyses of the *Wnt* genes have mainly focused on some phyla, such as chordates [5, 6], echinoderms [7], or insects [2, 8], but no exhaustive analysis at the scale of the bilaterian animals (Bilateria) has been conducted so far. As a consequence, the evolutionary history of the *Wnt* gene family remains elusive. Aiming at clarifying orthology relationships of *Wnt* subfamilies in Bilateria, we have investigated the *Wnt* gene content of two lophotrochozoan species, the annelid *Platynereis dumerilii* and the mollusc *Patella vulgata*. In addition, we have retrieved from genome and protein databases a comprehensive set of *Wnt* genes from various other metazoan phyla and have conducted rigorous phylogenetic analyses on this data set. In particular, the availability of the full sequence of the fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, and human genomes allows us to study the full set of *Wnt* genes that are present in these organisms.

Derivation of a Comprehensive Set of *Wnt* Genes in Bilateria

Amplification by PCR with degenerate primers yielded 320-pb fragments of six different *Wnt* genes from the annelid *Platynereis dumerilii* and four from the gastropod *Patella vulgata*. Vector-anchored RACE-PCR was used to isolate the full length (or at least larger fragments) of the corresponding genes (see the Experimental Procedures). We have aligned the Wnt domains of these newly obtained *Wnt* sequences to those of a large set of *Wnt* genes retrieved from databases.

We included in our alignment the 19 human, 7 fly, and 5 nematode *Wnt* genes that were retrieved from the corresponding genome projects and that are likely to represent the complete set of *Wnt* genes from these 3 species [2]. Preliminary phylogenetic analysis of the *Wnt* genes cloned from other vertebrates such as the mouse, zebrafish, and chick indicates that the 19 human genes are representative of the whole diversity of *Wnt* genes in vertebrates (not shown). Only human sequences were thus kept for most of the subsequent phylogenetic analyses. We also included in our alignment all the cloned amphioxus (as representatives of nonvertebrate chordates) and echinoderm (as representatives of nonchordate deuterostomes) *Wnt* genes. In addition, we also used several arthropod sequences (other than from *Drosophila*), a lophotrochozoan *Wnt* gene (from a brachiopod species), and the single known *Wnt* gene from a nonbilaterian animal (a cnidarian). The multiple alignment as well as a table of all the sequences (with their accession numbers) used in this work can be found in the Supplementary Material contained with this article online.

Evolutionary Relationships among Metazoan *Wnt* Genes

The multiple alignment was then used to construct phylogenetic trees of the *Wnt* gene family. We used three different methods of phylogenetic reconstruction: maximum parsimony (MP), maximum likelihood (ML, quartet puzzling), and a Bayesian phylogenetic inference approach, as described in the Experimental Procedures. Figure 1 shows a consensus of the trees obtained by the different methods (trees are supplied as Supplementary Figures). This consensus tree allows us to subdivide the *Wnt* gene family into paralogous subfamilies (originating by duplication), each subfamily containing orthologous *Wnt* genes (originating by speciation). We used both statistical support (bootstrap values, quartet puzzling support values, and posterior marginal probabilities) and congruence between the different phylogenetic methods as indicators of the reliability of the different subfamilies.

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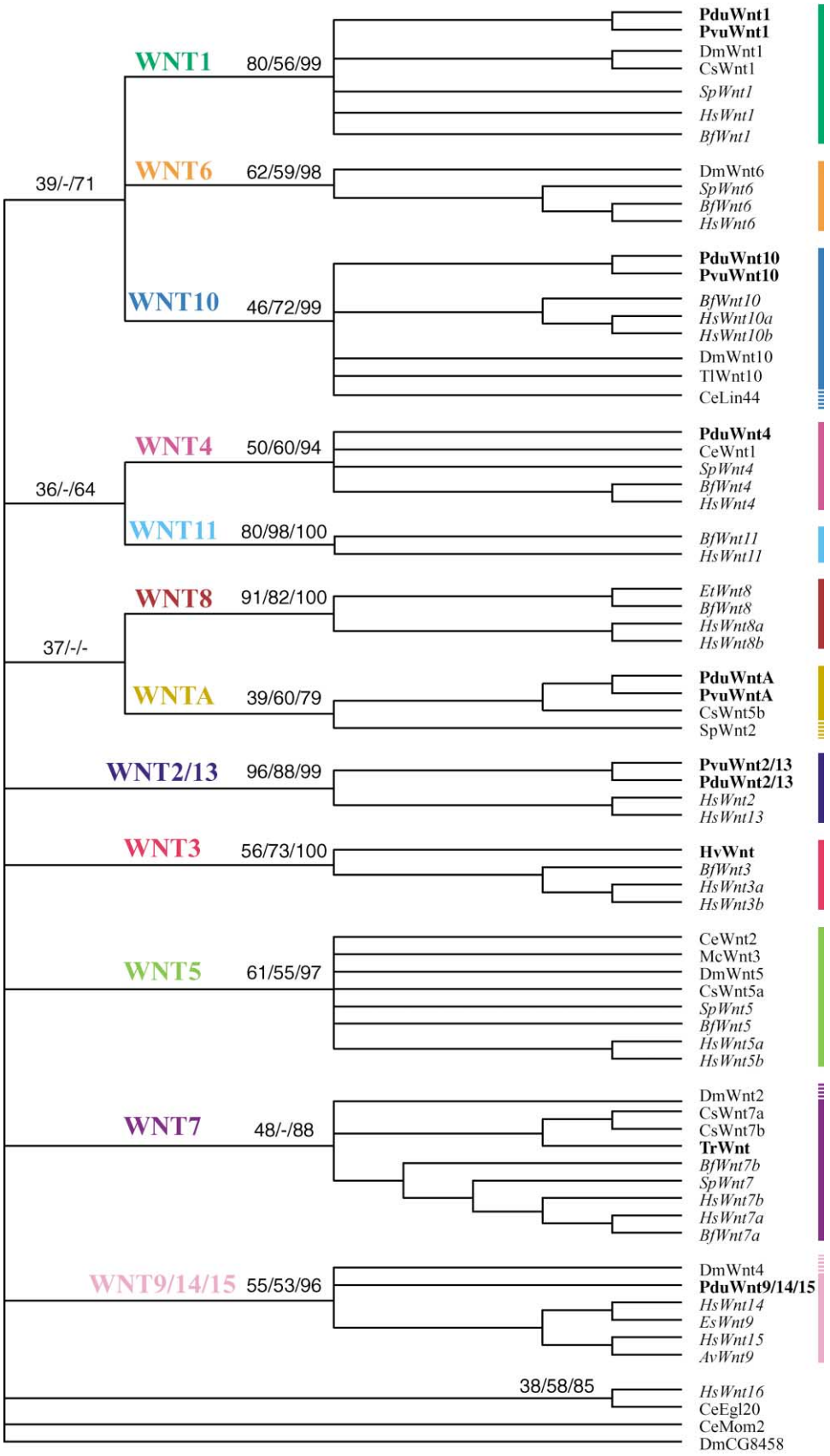
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80/56/99
WNT6
62/59/98
39/-/71
WNT10
46/72/99
WNT4
50/60/94
36/-/64
WNT11
80/98/100
WNT8
91/82/100
37/-/-
WNTA
39/60/79
WNT2/13
96/88/99
WNT3
56/73/100
WNT5
61/55/97
WNT7
48/-/88
WNT9/14/15
55/53/96
38/58/85
PduWnt1
PvuWnt1
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HsWnt10b
DmWnt10
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BfWnt11
HsWnt11
EtWnt8
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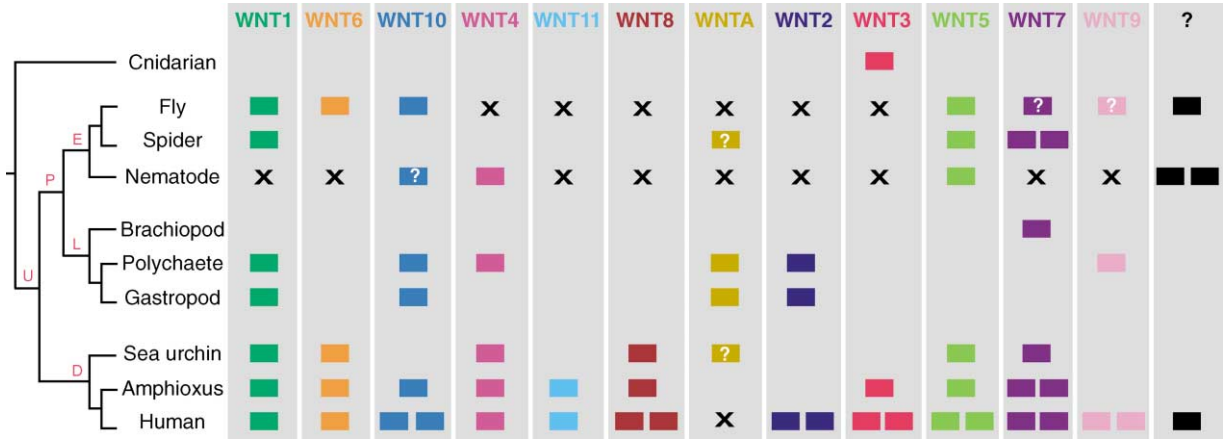

Figure 2. Distribution of *Wnt* Genes in Metazoans

The tree on the left summarizes the phylogenetic relationships [4] of the informative species used in this study. The last common ancestors of the main subdivisions of metazoans are shown: U, *Urbilateria*, bilaterian ancestor; D, deuterostome ancestor; P, protostome ancestor; E, ecdysozoan ancestor; L, lophotrochozoan ancestor. The different Wnt subfamilies are delineated by vertical white bars. Uncertain orthology relationships are indicated by question marks. A box indicates the presence of a member of a given Wnt subfamily in a particular group. An "X" indicates that no member of a Wnt subfamily is found in the fully sequenced genome of the corresponding organism. Obviously, regarding non-fully sequenced species, one cannot rule out the possibility that some other Wnt subfamily members are still to be found. The question mark in the upper right-hand corner of the chart represents orphan *Wnt* genes. The full names of the Wnt2 and Wnt9 subfamilies are Wnt2/13 and Wnt9/14/15, respectively.

We found 12 subfamilies that contain most of the *Wnt* genes (Figure 1). Eleven of these subfamilies have representatives in vertebrates, and, for that reason, we used the vertebrate nomenclature to name these different subfamilies (Wnt-1, -2/13, -3, -4, -5, -6, -7, -8, -9/14/15, -10, and -11). We investigated their evolutionary origin, given that the presence of orthologous sequences both in deuterostomes and protostomes (ecdysozoans and/or lophotrochozoans) implies that a representant of this subfamily was already present in the last common ancestor of the bilaterians, *Urbilateria* [9], and gave rise to the subfamily. Eight out of the 12 Wnt subfamilies fulfill this criterion: Wnt-1, -2/13, -4, -5, -6, -7, -9/14/15, and -10 (Figure 2). Following similar reasoning, the Wnt-3 subfamily, whose orthologs are found both in chordates and in the nonbilaterian hydra, originates before the divergence of bilaterians and cnidarians. Thus, at least nine *Wnt* gene subfamilies were already present in *Urbilateria* (Figure 2).

One subfamily (named Wnt-A to avoid confusion with other *Wnt* genes) contains lophotrochozoan, echinoderm, and spider Wnt genes (Figure 1). This subfamily may hence represent an additional Wnt type that is ancestral to Bilaterians. This would imply that members of this subfamily have been lost several times in both protostome and deuterostome lineages (as Wnt-A genes do not exist in chrodates, fly, and nematode). We have, however, to note that the association of the two closely related lophotrochozoan Wnt-A genes with the echinoderm and spider sequences is only poorly supported in our phylogenetic analyses (Figure 1). As both sea urchin and spider sequences consist of short PCR fragments, the confirmation of the ancestrality of the Wnt-A subfamily will require the isolation of a longer sequence. We also note that the Wnt-A subfamily has a tendency to cluster in our phylogenetic analyses with the Wnt-8 subfamily (which only contains deuterostomes genes). A simple hypothesis would hence be that Wnt-8 genes would represent divergent Wnt-A orthologs. Likewise, the Wnt-11 subfamily, which is only found in chordates, could represent an additionnal ancestral subfamily or a divergent chordate-specific duplication of the Wnt-4 subfamily (Figure 1).

In summary, our results point out the existence of 9–10 different types of *Wnt* genes in the last common ancestor of Bilateria. In addition, the fact that the single

Figure 1. A Consensus Phylogenetic Tree of the *Wnt* Gene Family

The results from the three phylogenetic reconstruction methods that have been used in this study (see the Experimental Procedures and the Supplementary Material) are summarized in this figure. Statistical support values of Wnt subfamilies found by the different methods (MP, ML, and Bayesian inference) are indicated on the corresponding node (bootstrap values, quartet puzzling support values, and posterior marginal probabilities, respectively). *Wnt* gene subfamilies are delimited by vertical colored lines on the right; the dotted portions of these lines indicate uncertain affiliation to the corresponding subfamily. The association between *HsWnt16* and *CeEgl20*, though statistically well supported in various methods, is highly suspect given the dissimilarity between these sequences and with other *Wnt* sequences. Hence, this clustering may be artifactual due to the "long branch attraction phenomenon". Species name abbreviations: *Av*, *Alopias vulpinus* (shark); *Bf*, *Branchiostoma floridae* (amphioxus); *Ce*, *Caenorhabditis elegans*; *Cs*, *Cupiennius salei* (spider); *Dm*, *Drosophila melanogaster*; *Es*, *Eptatretus stoutii* (hagfish); *Et*, *Evasterias troschelii* (echinoderm); *Hs*, *Homo sapiens*; *Hv*, *Hydra vulgaris*; *Mc*, *Mysidium columbiae* (crustacean); *Pdu*, *Platynereis dumerilii* (annelid); *Pvu*, *Patella vulgata* (mollusc); *Sp*, *Strongylocentrotus purpuratus* (echinoderm); *Tl*, *Triops longicaudatus* (crustacean); *Tr*, *Terebratulina retusa* (brachiopod). Deuterostome genes are italicized, lophotrochozoan genes are in bold, the single cnidarian gene is both italicized and in bold, and ecdysozoan genes are in regular type.

cnidarian sequence cloned to date clusters with the Wnt-3 subfamily rather than being orthologous to several bilaterian subfamilies strongly suggests that at least some of these subfamilies were already established in the last common ancestor of Cnidaria and Bilateria, i.e., earlier in metazoan evolution. We hence expect other *Wnt* genes to be found in cnidarians.

Evolution of *Wnt* Genes in Bilaterians

Several aspects of our analysis illuminate the evolutionary history of the *Wnt* gene family. First, there is no protostome-specific subfamily and only one or two subfamilies (Wnt-11 and maybe Wnt-8; see above) that are specific to deuterostomes or chordates (Figure 2). Moreover, most subfamilies (or even all if we consider *Wnt-A* as derived *Wnt-8* genes; see above) have representative genes in vertebrates, and the existence of these genes indicates that no Wnt type has been lost in the deuterostome lineage that leads to vertebrates. The large number of *Wnt* genes found in vertebrates (as seen by the 19 human genes), as compared to other phyla, is due to the fact that 7 subfamilies (Wnt-2/13, -3, -5, -7, -8, -9/14/15, and -10) contain 2 vertebrate members that are collectively orthologous to a single gene from other phyla (Figure 1). In most of these cases (five out of seven), a single ortholog is found in amphioxus (Figure 2), the sister group of vertebrates [6, 10], and the presence of this single ortholog indicates that these extra copies are the result of duplication having occurred during vertebrate evolution. This hence suggests that there were no important further duplications of the ancestral repertoire of *Wnt* genes at the base of the three main bilaterian lineages.

A second aspect of our work concerns ecdysozoans *Wnt* genes. A consequence of the above considerations is that there are fewer *Wnt* genes, both in the nematode and the fly, than in *Urbilateria*, and this indicates that specific *Wnt* gene losses have occurred in these groups. In addition, *Wnt* gene sequences from both nematode and fly are often quite divergent, usually making the identification of orthology relationships with other genes more difficult. We nevertheless were able to confidently relate three of the five nematode genes and six of the seven fly genes with vertebrate genes (Figure 2). In particular, we found Wnt-4, -5, and -10 genes in the nematode and Wnt-1, -5, -6, -7, -9/14/15, and -10 genes in the fly (Figure 1), which is partly in agreement with previous proposals [2, 5, 6, 8]. We have to note that some of these identifications (nematode Wnt-10 and fly Wnt-7 and -9/14/15) are subject to caution, as significant support was only found by Bayesian inference (Figure 1). The presence of a clear *Wnt*-7 in a spider (W. Damen, personal communication; Figure 1) confirms the existence of the Wnt-7 subfamily in arthropods. On the contrary, we do not find support for the identification of the fly sequence *DmCG8458* as a *Wnt-8* ortholog as was previously proposed, based on overall similarity (Figure 1) [2]. These results indicate that two or three Wnt subfamilies (Wnt-2/13, -3, and maybe -8/-A) have been lost in both fly and nematode, and possibly in all ecdysozoans. In addition, losses specific to nematode (Wnt-1, -2, -7, -9) or fly (Wnt-4) have also occurred (Figure 2).

A third aspect concerns our investigation of *Wnt* gene content in an annelid and a mollusc, which allows us to assess the relationships of lophotrochozoan *Wnt* genes with those of deuterostomes and ecdysozoans. Our analysis indicates the existence of Wnt-1, -2, and -10 as well as Wnt-A (possibly Wnt-8; see above) in both *Patella* and *Platynereis*, and Wnt-4 and -9/14/15 *Platynereis* members (Figure 2). These data confirm the existence of the Wnt-1, Wnt-4, and Wnt-10 subfamilies in the ancestor of bilaterians, but most importantly, the set of sequences isolated on *Platynereis* and *Patella* allows us to determine the ancestry of two subfamilies, Wnt-2/13, which lacks an ortholog outside vertebrates, and Wnt-9/14/15, which had only an ambiguous ortholog in fly (misleadingly named *DWnt4*, see above). Finally, the single brachiopod *Wnt* gene cloned to date clusters with the Wnt-7 subfamily and further strengthens the ancestry of the Wnt-7 subfamily at the scale of the bilaterians. Taken together, our data highlight the need of lophotrochozoan models in comparative genetics and developmental studies at the scale of the bilaterians, as it reveals unexpected classes of orthology ancestral to Bilateria, the representatives of which were lost or became very divergent in the ecdysozoan lineage.

A fourth aspect is about the relationships among the different Wnt subfamilies. Despite the fact that most of Wnt subfamilies are well statistically supported, there is not enough phylogenetic resolution to distinguish reliable relationships among most of the different Wnt subfamilies (Figure 1). The only exceptions are the clustering, on the one hand, of the Wnt-4 and Wnt-11, and, on the other hand, of the Wnt-1, -6, and -10 subfamilies. As discussed above, the Wnt-11 subfamily might have originated from a duplication of the Wnt-4 subfamily that would have occurred in the chordate lineage, i.e., very recently with respect to the origin of the different Wnt subfamilies, and the association of the two subfamilies probably only reveals a shorter time of divergence. The association of the Wnt-1, -6, and -10 subfamilies in our phylogenetic trees has to be considered in the light of the physical association of the corresponding genes into a cluster in the genome [2], a situation that favors processes such as genic conversion that would bias the phylogenetic signal. Alternatively, these phylogenetic associations may be due to shared functional specificities [11].

Conclusions

We have identified several new *Wnt* genes in two lophotrochozoan species, the annelid *Platynereis dumerilii* and the mollusc *Patella vulgata*. The phylogenetic analysis we conducted, including a broad sampling of *Wnt* sequences available from databases, unraveled unexpected orthology relationships. Altogether, our results allow the identification of 12 Wnt subfamilies in metazoans. Nine of them, at least, were already present in *Urbilateria*, the last common ancestor of all bilaterian animals.

Comparisons among extant species have given some insights into the morphological and genomic features of this ancient ancestor. From the morphological point of view, *Urbilateria* was probably a coelomate with ante-

rior-posterior and dorsoventral polarity; rudimentary appendages; some form of metamerism; a heart; sense organs, such as photoreceptors; and a complex nervous system (reviewed, e.g., in [12]). Genetically, it possessed numerous homeobox genes, among which were at least 7 Hox genes [13], several intercellular signaling pathways (TGF-β, Hedgehog, Notch, EGF), several Pax genes [14], at least 38 C_2H_2 zinc fingers [15], and 43 basic helix-loop-helix [16, 17] transcription factors. Our analysis further indicates that its genome contained at least nine different *Wnt* genes, confirming the genetic complexity of this ancestor, not only in terms of effector genes, but also among secreted signaling proteins.

Despite differential losses of *Wnt* genes in the various protostome lineages, most Wnt subfamilies contain both protostome and deuterostome genes. Some Wnt subfamilies could only be resolved once currently available data were combined with lophotrochozoan sequences, and this highlights the need for a lophotrochozoan model system for genetics and developmental comparative studies.

Experimental Procedures

Isolation of *Wnt* Genes from *Platynereis dumerilii* and *Patella vulgata*

Nested PCR was used to amplify 320-pb fragments of *Platynereis* and *Patella Wnt* genes. We used generic primers aimed to amplify any Wnt types. PCR was done either on cDNAs reverse transcribed from mRNA isolated from 48-hr *Platynereis* embryos, or on mass-zapped staged *Patella* cDNA libraries. The primer combinations were as follows: wg3w1/wg3c1 (or wnt10w1/Wntc1), followed by wg3w2/wg3c2. Degenerated primer sequences are: wg3w1, gartgya artgycayggnatg; wg3c1, rcarcaccartgraangtrca; wnt10w1, aartgyaar tgycaygg; wg3w2, ggiwsitgyacngtnmgnaaracntgytgg; and wg3c2, ckrtriccnckncrcarca. Six different *Wnt* genes were thus isolated from *Platynereis*, and four were isolated from *Patella*. We also used other degenerate Wnt subfamily-specific primers that failed to amplify additionnal sequences. Nevertheless, additional *Wnt* genes may exist in both species, as we only made our PCR screens on defined developmental stages. The 3′ and 5′ ends of some of the corresponding genes were amplified from staged cDNA libraries by using vector-specific primers and gene-specific (nondegenerate) primers. Primer sequences and experimental conditions are available upon request. PCR products were TA cloned into the PCR2.1 vector (Invitrogen) and were sequenced on an ABI automated sequencer.

Retrieval and Alignment of *Wnt* Gene Sequences

Wnt protein sequences were obtained through the retrieval of Wnt protein sequences listed on Roel Nusse's Wnt home page (http://www.stanford.edu/~rnusse/wntwindow.html; [2]) and the SMART database (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de), as well as by BLASTP search [18] at the National Center for Biotechnology. All sequences and their accession numbers are available as Supplementary Material (Table S2). Protein alignments were carried out by using ClustalW [19]. Only the Wnt domain, as determined in SMART and Pfam (Pfam = protein families database of alignment and HMMs, http://www.sanger.ac.uk/Software/Pfam/index.shtml), was used in our analyses, because the remaining part of proteins from different species are either not homologous or have diverged so much that the alignments are unreliable. The alignments were subsequently manually improved by using, as a guide, the alignments of Wnt domains available in Pfam and SMART. The Wnt domain itself contains several regions of high conservation separated by less-conserved stretches of amino acids that are not particularly well aligned. Given that sequence alignment influences phylogenetic reconstruction (reviewed in [20]), we explored alternative alignments of these less-conserved regions by changing the parameters of ClustalW, particulary the gap penalty. These different alignments gave essentially identical results in the phylogenetic analyses.

Phylogenetic Analyses

Unweighted maximum-parsimony (MP) reconstructions were performed with the PAUP 4.0 program [21]. The MP analysis was performed with the following settings: heuristic search of over 100 bootstrap replicates, MAXTREES set up to 2000, and other parameters set to default values. Maximum likelihood (ML) analyses were done with TreePuzzle [22]. The ML analysis was performed by using the quartet puzzling tree search procedure, with 25,000 puzzling steps. We used the Jones-Taylor-Thornton (JTT) model of substitution [23], the frequencies of amino acids being estimated from the data set, and let rate heterogeneity across sites to be modeled by two rate categories (one constant and four γ rates) [22].

Quartet puzzling is only an approximation to the method of maximum likelihood (ML), for which exact implementations also exist (proposed, for instance, by the program PAML) [24]. It has been shown that quartet puzzling does not always perform as well as exact ML methods and is not immune to artifacts [25]. On the other hand, ML estimation is computationally infeasible, in the present case, because of the size of the data set. Bayesian inference turns out to be much more efficient than ML methods, while giving roughly equivalent results. Larget and Simon [26] developed Bayesian tools for the phylogenetic analysis of nucleotide sequences (Bambi). Here, we have used an adaptation of these tools aimed to handle amino acid sequences (N.L., unpublished data). This implementation allows for rate variations across sites using a flat Dirichlet prior, like in Bambi, and takes into account substitution profile heterogeneities across sites, by using a Dirichlet process mixture model [27]. Two independent Markov chains were run, each containing 16,000,000 Monte Carlo steps, after a burn-in of 2,000,000 steps. One out of every 8000 trees was saved. For each run, a sample of 2000 trees was thus collected, of which a majority consensus was built. Marginal probabilities at each node were taken as a measure of statistical support. The average discrepancy between the estimated probabilities obtained in the two runs is 3% on average and never exceeded 10%, except at the base of the Wnt-1/-6/-10 group, for which the values 63% and 78% were observed. The results obtained from the two runs are thus consistent, so that we finally combined them by gathering the 4000 trees of the two samples. In addition, we observed throughout the samples that the variable positioning of one particular sequence, *CeMom2*, tends to disrupt the resolution of a few nodes that are otherwise stable. This led us to first eliminate the terminal branch leading to *CeMom2* in each of the 4000 trees before computing the consensus. It should be stressed that, by using this procedure, we only change which part of the total information extracted from the data we decide to display, while leaving the probabilistic inference completely invariant.

Trees were displayed with PAUP 4.0 (or TreeView [28] for the ML tree), saved as PICT files, converted into JPEG files with Graphic Converter, and then annotated using Adobe PhotoShop and Illustrator.

Supplementary Material

Supplementary Material including the phylogenetic trees (Figures S1–S3) on which Figure 1 is based, as well as a multitude alignment of all sequences used to construct these trees (Table S1) and a list of these sequences with their accession numbers (Table S2) is available at http://images.cellpress.com/supmat/supmatin.htm.

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Accession Numbers

The sequences derived from the TA-cloned PCR products have been submitted to the EMBL nucleotide database as accession numbers AJ491796–AJ491805.