A Phylogenetic Tree of the \textit{Wnt} Genes Based on All Available Full-Length Sequences, Including Five from the Cephalochordate Amphioxus

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The \textit{Wnt} gene family is large, and new members are still being discovered. We constructed a parsimony tree for the \textit{Wnt} family based on all 82 of the full-length sequences currently available. The inclusion of sequences from the cephalochordate amphioxus is especially useful in comprehensive gene trees, because the amphioxus genes in each subfamily often mark the base of the vertebrate diversification. We thus isolated full-length cDNAs of five amphioxus \textit{Wnt} genes (\textit{AmphiWnt1}, \textit{AmphiWnt4}, \textit{AmphiWnt7}, \textit{AmphiWnt8}, and \textit{AmphiWnt11}) for addition to the overall \textit{Wnt} family tree. The analysis combined amino acid and nucleotide sequences (excluding third codon positions), taking into account 97\% of the available data for each sequence. This combinatorial method had the advantage of generating a single most-parsimonious tree that was trichotomy-free. The reliability of the nodes was assessed by both jackknifing and Bremer support (decay index). A regression analysis revealed that branch length was strongly correlated with branch support, and possible reasons for this pattern are discussed. The tree topology suggested that in amphioxus, at least an \textit{AmphiWnt5} and an \textit{AmphiWnt10} have yet to be discovered.

Introduction

Two recent research trends are influencing evolutionary reconstructions within families of important developmental genes (like \textit{Hox} and \textit{Pax}). First, the phylogenetic sampling of taxa is rapidly broadening, and second, the superphyletic groupings of animal phyla are undergoing substantial modification (Finnerty and Martindale 1998; de Rosa et al. 1999). One of the most prominent gene families comprises the \textit{Wnt} genes, which encode secreted signaling proteins with a wide diversity of developmental roles. Existing considerations of the evolutionary history of the \textit{Wnt} genes are out of date (Sidow 1992; Graba et al. 1995; Blader, Strähle, and Ingham 1996), are not based on full-length sequences (Ferkowicz, Stander, and Raff 1998), or cover only selected sequences (Sasakura, Ogasawara, and Makabe 1998).

The purpose of the present paper was to use the large \textit{Wnt} data set now available to reexamine the phylogenetic history of this gene family. A special feature of our phylogenetic analysis is the inclusion of five recently discovered \textit{Wnt} genes from the cephalochordate amphioxus (Holland, Holland, and Schubert 2000; Schubert, Holland, and Holland 2000a, 2000b; Schubert et al. 2000). Amphioxus genes in general are typically very similar to their homologs in vertebrates, but often serve as useful markers for the base of the diversification of vertebrate sequences in gene trees (e.g., Shimeld 1997; Kozmik et al. 1999).

The \textit{Wnt} tree presented in this paper is based on all 82 currently available full-length sequences, including 5 from amphioxus. Wnt proteins typically comprise about 350–400 amino acids and are defined by about 100 conserved amino acid residues (including 23 or 24 distinctive cysteines) distributed across the entire protein exclusive of a short N-terminal leader sequence (Nusse and Varmus 1992). In general, the C-terminal half of Wnt proteins shows the highest degree of sequence conservation, with most of the incomplete \textit{Wnt} clones isolated to date corresponding to this region.

Approximately 97\% of each full-length \textit{Wnt} sequence was alignable and used for the analysis. In order to construct a phylogenetic tree from this large data set, we used a parsimony method based on a combination of the nucleotide and amino acid sequences (Agosti, Jacobs, and DeSalle 1996), as modified to exclude third codon positions (Jacobs et al. 1998). We assessed the robustness of tree topologies both by jackknifing and by Bremer support (decay index). With the exception of \textit{AmphiWnt1}, the amphioxus \textit{Wnt} genes were situated just basal to the duplication of vertebrate genes in the respective subfamilies. As inferred from the distribution of genes in each subfamily, additional amphioxus \textit{Wnt} genes yet to be found probably include \textit{AmphiWnt5} and \textit{AmphiWnt10}. For the \textit{Wnt} tree as a whole, the structure of most of the individual subfamilies was strongly supported, but the branching patterns deeper in the tree were more problematical. It is likely that the deep branching pattern of the overall \textit{Wnt} tree will be revealed more robustly only when more full-length sequences become available for nonarthropod protostomes, lower deuterostomes, and prebilaterians.

Materials and Methods

Phylogenetic Tree Construction and Analysis

The analysis included amino acids and nucleotides of all 82 full-length \textit{Wnt} clones that were available from BLAST searches of Internet databases through the end of 1999. \textit{Wnt9} and \textit{Wnt15} subfamilies have been discovered, but so far they are known only from incomplete clones and thus were not considered in the analysis. Accession numbers are listed in table 1 of Schubert, Holland, and Holland (2000a) except for \textit{AmphiWnt1} (AF061974), \textit{AmphiWnt8} (AF190470), and \textit{AmphiWnt11} (AF442813).
We used the following procedures to assess the sensitivity of the data to issues of alignment and long-branch attraction: (1) Caenorhabditis sequences, which tend to be difficult to align, were excluded; (2) only deuterostome taxa were included; (3) only vertebrate taxa were included; and (4) the relatively diverse 5' ends of the sequences were excluded entirely or were arbitrarily weighted by 0.1 or 0.3. These approaches generated alternative topologies in some of the poorly supported basal nodes of the tree, but none of these alternatives was supported by strong Bremer or jackknife values (data not shown).

In addition, we conducted a set of statistical regression/correlation analyses relating terminal branch length to branch support with special reference to Caenorhabditis sequences. The three sea urchin sequences were omitted because some of the relatively variable 5' sequence was not available. These analyses took into consideration lengths of terminal branches (fig. 1), branch support as measured by jackknife values (fig. 2), estimates of branch divergence times, and fidelity of taxonomic placement of a given gene within its subfamily. Examples of branch divergence times were 60 Myr for mice and humans (Benton 1997; McKenna and Bell 1997; Foote et al., 1999), 400 Myr for fish and mammals, 600 Myr for amphioxus and vertebrates, and 600 Myr for taxa not clearly associated with any Wnt subfamily (assuming their divergence correlates with the Cambrian radiation). Taxonomic fidelity of a given sequence was scored according to closeness of fit with accepted taxonomy and apparent gene duplication events as follows: rank 1, congruence with traditional taxonomy; rank 2, minor incongruence within a taxonomic class; rank 3, not congruent with higher-level taxonomy, but assignable to a Wnt subfamily; rank 4, not assignable to a subfamily with other Wnt genes.

Results and Discussion

Overall Tree of the Wnt Family

The single most-parsimonious tree based on 82 full-length Wnt genes is shown in figure 1 (in which the relative branch lengths emphasize the degree of relatedness between the branches) and figure 2 (which shows the support values for the branches). The overall tree is large but free of polychotomies, and many of the nodes are supported by robust jackknife values and robust Bremer support values. In general, branching patterns within subfamilies are more strongly supported than relationships between the subfamilies, as manifested by decreasing jackknife and Bremer numbers toward the base (left part) of the tree. The subfamilies will be considered in order, according to whether their internal branching orders were relatively robust (Wnt3, Wnt4, Wnt7, Wnt10, Wnt11), fair (Wnt1, Wnt2/13, Wnt5, Wnt8), or poor (Wnt6, Wnt14, Wnt16).

Branch Length and Branch Support Analyses

The analysis of the overall Wnt tree described above tended to give strong support within subfamilies but weak support for the relations among subfamilies. We used the following procedures to assess the sensitivity of the data to issues of alignment and long-branch attraction: (1) Caenorhabditis sequences, which tend to be difficult to align, were excluded; (2) only deuterostome taxa were included; (3) only vertebrate taxa were included; and (4) the relatively diverse 5' ends of the sequences were excluded entirely or were arbitrarily weighted by 0.1 or 0.3. These approaches generated alternative topologies in some of the poorly supported basal nodes of the tree, but none of these alternatives was supported by strong Bremer or jackknife values (data not shown).

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Rationale for Combinatorial Methods to Determine Tree Topology and Support

Because the Wnt gene family represents a large data set, the present analysis was based on a combination of both nucleotide and amino acid sequences, and support of the nodes was evaluated by a combination of jackknifing and Bremer support. Thus, within an overall gene tree of the Wnt family, relationships ranged from close (e.g., mouse versus rat) to distant (e.g., mouse versus Drosophila). The combination of nucleotide-coding and amino acid–coding characters was justified because, due to the nature of the genetic code, nucleotides and amino acids are partially independent (Agosti, Jacobs, and DeSalle 1996; Jacobs et al. 1998). When the two
types of character codes are both informative and in agreement, this information was accorded the greatest weight; when only one of the character codes was informative, it received an intermediate weight; and when the two character codes were in conflict, the characters received the least weight. As discussed in detail by Jacobs et al. (1998), the first two nucleotides of each codon are relatively conservative and useful in defining basal branches of the tree. Only those changes at the third position that alter an amino acid can influence the tree topology.

The reliability of a combinatorial analysis was assessed by analyzing the amino acid and nucleotide data independently. The 48 most-parsimonious amino acid trees and the 5 most-parsimonious nucleotide trees recovered from the analyses, respectively, yielded an ami-
no acid consensus tree and a nucleotide consensus tree, respectively (data not shown). There were eight polychotomies in the amino acid–based tree and two in the nucleotide-based tree. In contrast, the higher resolution of the trees based on a combination of amino acids and nucleotides (figs. 1 and 2) was indicated by the absence of any polychotomies. From one analysis to the next (amino acids alone, nucleotides alone, or a combination of both), there was little difference between topologies within subfamilies, but considerable diversity among subfamilies, as would be expected from the low values supporting the more basal nodes (fig. 2).

Jackknifing and bootstrapping are both resampling methods, but for large data matrices like the one in the present paper, the former is certainly faster and probably more stringent than the latter (Farris et al. 1996).
cause resampling methods in general are sensitive to problems of character independence and distribution (Page and Holmes 1998), we also calculated Bremer support values for each node in the tree. Such a combination of resampling along with a decay index method for the same data is becoming more common in phylogenetic analyses (e.g., Jondelius 1998; Littlewood, Rhode, and Clough 1999). Bremer support is based on the number of extra steps required to lose a branch from the consensus tree of near-most-parsimonious trees. Unlike jacksonifying, Bremer support does not involve manipulation of the data to simulate random reweighting. In the present study, it is possible that the jacksonknife values may be sensitive to the combination of data for amino acids and nucleotides. Therefore, it is reassuring that the robust jacksonknife values usually correlate with robust Bremer support values (fig. 2).

Low jacksonknife and Bremer support values are usually correlated in figure 2, especially in basal branches of the tree or where long-branch attraction is likely. Conversely, high Bremer support is usually correlated with high jacksonknife percentages at the more terminal branches within each Wnt subfamily. Occasionally, low Bremer support is correlated with a high jacksonknife percentage, e.g., at the node separating the Wnt1 subfamily from the rest of the tree and at the base of the vertebrate Wnt10 sequences. Similar but less extreme examples can be observed at three branches within the Wnt2/13 subfamily and at two branches within the Wnt8 genes. In all of these mismatches, the low Bremer support values presumably reflect the higher stringency of this decay index method. In contrast, high Bremer support combined with very low jacksonknife values is not conspicuous anywhere in the tree.

Correlation of Branch Length and Branch Support in the Overall Wnt Tree

It would have been artistically awkward to present branch length data (fig. 1) with branch support data (fig. 2) in a single figure. Even so, in comparing the two figures, one gets the impression that long branches are correlated with low support (this exemplifies a general problem especially likely to be encountered in taxon-rich phylogenetic analyses). We verified this pattern by a multistep regression analysis between terminal branch length and branch support and considered possible reasons for their correlation. Both features are influenced by divergence time, so both variables were regressed against divergence time to yield residuals that were not directly a product of divergence time. These residuals were subsequently regressed against each other to assess the time-independent influence of branch length on branch support. The initial regression of branch length versus divergence time revealed a nonlinearity with disproportionately high branch lengths for the 600-Myr cohort. This discrepancy has been previously observed in similar data and attributed to the early divergence of Cambrian taxa (Wray, Levinton, and Shapiro 1996) or to rate variation between vertebrates and invertebrates (Ayala, Rzhetsky, and Ayala 1998). We obtained a better fit to our data with a polynomial regression ($R^2 = 0.823; P < 0.0001$). Jackknife values regressed against divergence time generated a strong negative relationship, as expected ($R^2 = 0.439; P < 0.0001$). Regression of the residuals of branch length versus the residuals of jackknife support generated a significant regression ($R^2 = 0.153; P = 0.0005$) with a negative slope, demonstrating a time-independent negative relationship between terminal branch length and branch support. Our metric of taxonomic fidelity was also significantly correlated with both residuals of branch length and residuals of branch support in Kendall rank correlation analyses, documenting less fidelity with increased branch length and with reduced jacksonknife support.

In the present study, in common with phylogenetic analyses generally, Caenorhabditis sequences tended to be associated with long branches. Therefore, it was instructive to compare just the Caenorhabditis and amphioxus data from the 600-Myr cohort. All amphioxus sequences have a taxonomic fidelity of 1, with the exception of AmphiWnt1, which has a fidelity of 3. In contrast, all the Caenorhabditis sequences have a fidelity of 4. Moreover, the branch lengths of the five amphioxus sequences average 185, as compared with an average branch length of 296 for the five Caenorhabditis sequences. In sum, there was a negative relationship between branch length and branch support that was largely independent of divergence time, and Caenorhabditis sequences consistently had disproportionately long branches. These long branches likely resulted from some combination of the following: (1) high rates of evolution in Caenorhabditis and/or other protostomes; (2) limited sampling of protostome taxa, resulting in reduced precision of alignment; and/or (3) the necessarily long branches resulting in phylogeny reconstruction with a lower density of taxon sampling, as is evident from the protostome Wnt data.

Subfamilies with Robust Internal Branch Support

The Wnt3 family as a whole was strongly supported (11/100) and comprised only vertebrate sequences that bifurcated into a Wnt3 cluster and a Wnt3a cluster. A Wnt3 gene has also been found in an echinoderm but is known only from a partial sequence and was thus not included in the present analysis. If this echinoderm sequence is taken into account, it is possible that Wnt3 genes are characteristic of deuterostomes in general.

The Wnt4 subfamily, which comprises only deuterostomes, was very well supported (11/100) with the following branching order: echinoderms, amphioxus, vertebrates. Within the vertebrates, the chicken sequence unexpectedly grouped more closely to the Xenopus sequence than to the mouse sequence, as was previously noted by Hollyday, McMahon, and McMahon (1995). The topology of this part of the tree would probably be improved by addition of more sequences from lower deuterostomes and agnathans.

The well-supported (8/96) Wnt7 subfamily had the following branching order: amphioxus, vertebrates. Within the vertebrate sequences, support values were
very robust. In addition to the sequences in the alignment, an incomplete but probable Wnt7 sequence is known for an echinoderm. Amphioxus AmphiWnt7 branches off the tree before diversification into Wnt7a and Wnt7b within the vertebrates. The exact origin of this duplication event remains to be revealed by further studies of Wnt7 genes in lower deuterostomes.

The basis of the Wnt10 genes was established by two weakly supported protostome sequences (Drosophila Wnt2 and Caenorhabditis Lin44) flanking a well-supported vertebrate group. This association could reflect long-branch attraction. Within the vertebrate Wnt10 sequences, there was evidently a gene duplication basal to the early gnathostomes to produce Wnt10b (formerly called Wnt12) and Wnt10a clades. Further description of Wnt10 sequences will help to elucidate whether this subfamily is restricted to vertebrates or whether there are also invertebrate homologs, including an AmphiWnt10 in amphioxus.

In the Wnt11 subfamily, most branches were very well supported. The branching order (amphioxus, anamniote vertebrates, amniote vertebrates) was congruent with commonly accepted phylogenies based on morphological characters. The only exception was the weakly supported (3/51) inverted order of the zebrafish and Xenopus Wnt11. The obvious duplication of an ancestral zebrafish Wnt11 into Wnt11a and Wnt11b probably reflects telost-specific genome duplications. The addition of basal vertebrate Wnt11 sequences might help to resolve this part of the tree.

Subfamilies with Fair Internal Branch Support

The Wnt1 (also called wingless in insects) subfamily is represented by both protostome and deuterostome sequences. Caenorhabditis Wnt1 was highly divergent, although still more closely related to the other Wnt1 genes than to any other Wnt subfamily. Moreover, AmphiWnt1 from amphioxus was not located at the base of the vertebrates as would be expected, but instead fell basal to a clade including both protostome and deuterostome Wnt1 sequences. The branching order within the Wnt1 clade (arthropods, invertebrate deuterostomes, anamniote vertebrates, amniote vertebrates) was congruent with generally accepted phylogenetic schemes based on morphological characters. Resolution at the base of the Wnt1 subfamily can be expected to improve when Wnt1 genes are described for additional taxa, especially lower deuterostomes and agnathan vertebrates. The outlying position of AmphiWnt1 was not altered when the Wnt1 subfamily was analyzed in isolation or only insect Wnt1 genes were used as the outgroup to the Wnt tree as a whole. When analysis was based only on the relatively conserved C-terminal two-thirds of the Wnt1 protein sequences (Holland, Holland, and Schubert 2000), AmphiWnt1, although grouping with other deuterostome Wnt1 sequences with weak branch support, still did not branch off at the base of the vertebrate diversification.

The Wnt2/13 subfamily, which was supported by maximal Bremer and jackknife values, was subdivided into a Wnt2 group and a Wnt2b/13 group, both with branching patterns consistent with morphology-based phylogeny. The Wnt2 and Wnt13 genes were so closely related that the terminology might be improved if the Wnt13 sequences were renamed Wnt2b. All of the known full-length sequences for Wnt2 and Wnt13 are from vertebrates. Although a partial Wnt2 sequence has been reported for an echinoderm (Sidow 1992), its identification as such is suspect because, over the most conserved 115 amino acids, only 35% are identical with mouse Wnt2 (as compared with 67% identity between the same region of mouse Wnt2 and mouse Wnt13). Thus, Wnt2 and Wnt13 might represent vertebrate-specific genes.

The Wnt5 subfamily had both vertebrate and invertebrate representatives, with the latter including a urochordate, an echinoderm, Drosophila, and Caenorhabditis (disconcertingly, the Caenorhabditis gene is named Wnt2). This subfamily was very poorly supported by Bremer and jackknife values, and its topology may have been a consequence of long-branch attraction. Addition of more invertebrate sequences would probably help group the invertebrate sequences basal to the vertebrate clade. With the echinoderm Wnt5 and Xenopus Wnt5c sequences at its base, the vertebrate branch bifurcated into Wnt5a and Wnt5b, presumably as a result of a gene duplication. The Wnt5a clade was well supported and had a branching order congruent with accepted vertebrate phylogeny. The Wnt5b clade, in spite of lower support values, showed an expected phylogeny (branching order: fish, amphibians, mammals); this pattern, previously noted by Blader, Strähle, and Ingham (1996), implies that zebrafish Wnt5 should be renamed Wnt5b. The location of Xenopus Wnt5c just basal to vertebrate Wnt5a and Wnt5b may reflect a duplication of this gene before the amphibian-mammal divergence. The existence of echinoderm and tunicate Wnt5 genes suggests that more invertebrate deuterostome Wnt5 genes, including a possible amphioxus AmphiWnt5, might still be described.

The maximally supported Wnt8 subfamily was represented by deuterostome sequences only. In addition to the analyzed sequences, a partial Wnt8 clone is known from an echinoderm. Amphioxus AmphiWnt8 branched off basal to the diversification of vertebrate Wnt8 and Wnt8b. Generally, the nodes within this subfamily were well supported, and the branching order of amphioxus, zebrafish, Xenopus, amniote vertebrates in both the Wnt8 and Wnt8b branches was congruent with accepted vertebrate phylogeny. The amniote vertebrate part of the Wnt8 branch was weakly supported (2/45) and had confusing nomenclature, suggesting that additional vertebrate Wnt8 sequences could help to resolve the phylogeny of this part of the tree.

Subfamilies with Poor Internal Branch Support

In the Wnt6 subfamily, only one full-length sequence has been described (from the mouse). However, several incomplete but likely Wnt6 sequences are known in other vertebrates, in echinoderms (Sidow 1992), and...
also in amphioxus (Holland et al. 1994). Description of more representatives of the Wnt6 subfamily will help to resolve this part of the tree. In all of the less-inclusive analyses (see Materials and Methods), as well as in the full-length analysis detailed here, mouse Wnt6 consistently branches off just basal to the Wnt10 clade, indicating that this relationship might be more robust than the low Bremer and jackknife values (2/2) indicate.

The Wnt14 genes were represented by only two sequences, chicken Wnt14 and Drosophila Wnt4 (a partial human Wnt14 gene has been described). This part of the tree was poorly supported by both Bremer and jackknife values. The topology was probably deceptive due to long-branch attraction. More representatives of this subfamily are needed to establish the proper phylogeny and grouping of this subfamily.

The Wnt16 subfamily was represented only by one amniote vertebrate (human Wnt16) sequence and two protostome sequences (Caenorhabditis F38E1.7/MOM2 and W08D2.1/egl20). This subfamily was very poorly supported by Bremer and jackknife values, probably because of long-branch attraction. More full-length Wnt16 sequences will be necessary to clarify the topology of this part of the tree.

Conclusions

The methods used here can be expected to be generally useful for constructing phylogenetic trees from large genetic data sets. The combination of the nucleotide and amino acid data resulted in a single most-logon tree. The methods used here can be expected to be generally useful for constructing phylogenetic trees from large genetic data sets. The combination of the nucleotide and amino acid data resulted in a single most-plexing and Bremer support proved to be an effective way to highlight the ambiguous regions.

Some predictions are possible from the Wnt family tree. First, it is likely that more amphioxus Wnt genes remain to be discovered (most probably an AmphiWnt5, and possibly an AmphiWnt10). Because of the poor sampling of Wnt genes among the protostomes, the number of Wnt gene subfamilies in the ancestor of the Bilateria remains uncertain. The minimal number is three, as judged from the presence of three well-supported subfamilies (Wnt1, Wnt5, and Wnt10) that include sequences from both protostomes and deuterostomes (figs. 1 and 2). However, it would not be surprising to find a larger number of ancestral Wnt subfamilies when a wider spectrum of invertebrate phyla has been sampled. Finally, during early vertebrate evolution, the number of Wnt genes increased markedly.

For some Wnt subfamilies, only a few vertebrate representatives were included in the analysis (e.g., Wnt6, Wnt14, and Wnt16). Those branches were characterized by low support values and might suffer long-branch attraction. The proper phylogeny and the question of protostome or amphioxus representatives within these subfamilies can only be assessed if new genes of these groups are described. The present study also emphasizes that many of the available full-length Wnt sequences are from either insects or vertebrates, as are most of the partial Wnt clones currently known. In the future, in order to improve the Wnt gene phylogeny, it will be important to characterize Wnt genes from a much wider spectrum of animals, especially the noninsect protostomes.

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LITERATURE CITED


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