

# Hierarchical phylogenetics as a quantitative analytical framework for evolutionary developmental biology

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## Summary

Phylogenetics has inherent utility in evolutionary developmental biology (EDB) as it is an established methodology for estimating evolutionary relationships and for making comparisons between levels of biological organization. However, explicit phylogenetic methods generally have been limited to two levels of organization in EDB—the species and the gene. We demonstrate that phylogenetic methods can be applied broadly to other organizational levels, such as morphological structures or cell types, to identify evolutionary patterns. We present examples at and between different hierarchical levels of organization to address questions central to EDB. We argue that this application of “hierarchical phylogenetics” can be a unifying analytical approach to the field of EDB. *BioEssays* 27:1158–1166, 2005.

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## Introduction

The field of evolutionary developmental biology (EDB), or evo-devo, aims to understand the evolution of the developmental processes underlying phenotype. Achieving this aim requires examination of evolutionary changes at multiple levels of biological organization, from nucleotides, genes and cells to genetic networks, organs and species. In fact, many of the central questions in EDB hinge on understanding how historical changes at one level of organization relate to changes at other levels of organization.<sup>(1)</sup> For example, are particular developmental or genetic changes correlated with

phenotypic changes during evolution?<sup>(2–4)</sup> What is the tempo of gene expression evolution?<sup>(5–8)</sup> To what extent are gene duplications associated with diversification of genetic networks, organs or species?<sup>(9,10)</sup> What role does gene recruitment and co-option play in the evolution of novelty?<sup>(11–13)</sup> Such historical comparisons are inherently phylogenetic in nature.

Although phylogenetics already has a presence in EDB,<sup>(13–18)</sup> that presence is mostly focused on only two levels of biological organization—gene phylogenies and species phylogenies. Typically, gene trees have been used to identify gene family membership, while species trees have been used to examine the evolution of developmental characters, such as gene expression patterns. Researchers also implicitly compare gene trees to species trees to identify gene duplication or loss across taxa. Here we point out that explicit phylogenetic methods can also be used at other levels of biological organization, besides genes and species. This realization indicates that entire suites of established phylogenetic/comparative tools—such as character mapping and reconciled trees—can be used in new ways in EDB to test and generate general evolutionary hypotheses that are central to the field.

## Phylogenetics at new biological levels

Although most often focusing on genes and species, researchers are now beginning to conduct phylogenetic analyses on units from other levels of biological organization.<sup>(19–24)</sup> These studies either implicitly or explicitly use phylogenetic methods to generate trees of morphological structures,<sup>(20,21,24)</sup> cell types,<sup>(19)</sup> developmental fields,<sup>(22)</sup> or metabolic pathways.<sup>(23)</sup>

One structure that has received considerable attention is the eye. Oakley<sup>(21)</sup> discusses how this complex organ can be replicated and subsequently modified or lost. He argues this pattern of evolution can be reconstructed phylogenetically as an “eye tree”, where differences in eye structure or gene expression can identify “eye families” of diverse morphological types. Liu and Friedrich<sup>(22)</sup> extend the “eye tree” concept. They place eye developmental fields in a phylogenetic context and present evidence for subdivision of the eye field as a

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Funding agency: J.M.S. was supported by the University of California President's Postdoctoral Fellowship Program. T.H.O. was supported by a National Science Foundation Grant (DEB-0316330).

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DOI 10.1002/bies.20291

Published online in Wiley InterScience (www.interscience.wiley.com).

mechanism for duplication. This “developmental field tree” of eyes is then used to understand changes in developmental mechanisms that correspond to differences in insect larval and adult compound eyes.

Trees can also be constructed from elements at lower levels of biological organization, such as cells. Arendt<sup>(19)</sup> suggests that comparing cell types using molecular characters can provide insight into their diversification and evolution, and should be the focal point of EDB analysis. This cellular focus in EDB also has been championed by others.<sup>(25,26)</sup> Arendt<sup>(19)</sup> refers to this as “comparative molecular cell biology” and uses the approach to examine evolutionary relationships among vertebrate retinal cell types. In that study, similarities in the gene expression data across cell types were treated as homologous traits (characters), suggesting a single evolutionary history with a common ancestral cell type that diversified into the extant cell types. Although Arendt<sup>(19)</sup> did not treat gene expression data as explicit characters and character states for phylogenetic analysis, his goal, to generate evolutionary relationships among retinal eye types, was phylogenetic in nature: he envisioned a phylogenetic tree of retinal cell types.

Geeta<sup>(20)</sup> goes a step farther by explicitly analyzing structures phylogenetically. She demonstrates that a structure tree can be estimated from the variation of morphological characters during the evolution of seed plants. She then compares the evolutionary patterns between two organizational levels: the leaf primordium tree to the angiosperm species tree. Although the primary goal of Geeta’s paper was to determine “individuality” (= modularity) of leaf primordium as a “developmentally integrated structure”, she also demonstrates two other important points. First, she shows that it is possible to reconstruct the evolutionary relationships of morphologically variable structures and represent them as a branching pattern. Second, she illustrates the usefulness of multiple level comparisons that incorporate phylogenetic analysis to address EDB questions.

Like Geeta,<sup>(20)</sup> Coates et al.<sup>(24)</sup> explicitly generate a phylogeny of organismal structures using morphological data. The resulting tree estimates the historical relationship of morphological characters distributed between a serially repeated structure found within single organisms: pectoral and pelvic appendages of the appendicular skeleton in early tetrapods. Only by reconstructing the phylogeny of these appendages can the authors address an important concept in EDB: the influence of developmental integration. This is an important point, as developmental integration can only be understood in phylogenetic terms by simultaneously comparing two levels of biological organization.

Phylogenetic trees can also be constructed for biological networks. For example, Cunchillos and Lecointre<sup>(23)</sup> explicitly estimate the phylogenetic history of metabolic pathways. This framework provides the means to address questions involving

metabolism evolution, such as the relative timing of glycolysis and amino acid biosynthesis origins.

These papers demonstrate the potentially broad applications of phylogenetic methods in EDB that go beyond gene and species trees. Researchers are applying phylogenetic concepts both implicitly and explicitly at multiple levels of organization, illustrating the need for quantitatively estimated trees to address questions about evolutionary history. Therefore, we advocate here that elements at all organizational levels can be treated under an explicit phylogenetic framework providing insight into the evolutionary history of elements through tree construction, explicit tests of character homology, and an impartial analytical method.

From the studies described above, it is clear that there is a need to reconstruct the evolutionary history at many levels of organization, but what biological units can be examined under phylogenetic framework? To identify these elements, we can use a modular view of the organism, a central theme to EDB.<sup>(27–29)</sup> By definition, modules<sup>(11)</sup> are the distinct units that act cooperatively in an organism, but have a degree of dissociability due to their internal integration.<sup>(11,30–35)</sup> We can reconstruct the history of modules, such as genes, cell types and organs, because they are evolutionary lineages: they are individuals<sup>(36)</sup> that are spatiotemporally bounded in evolutionary time, with discrete origins, histories and extinctions.<sup>(36–38)</sup> As genetically coherent units, modules are heritable and dynamic.<sup>(11,28,31,33,39)</sup> They may vary during ontogeny or transform over evolutionary time as their internal components accumulate change.<sup>(28,34)</sup> As a result of this evolvability,<sup>(40)</sup> modules at all levels of biological organization have a distinct evolutionary history of “descent with modification” that can be represented as a tree diagram with a series of lineages (branches) diverging from a common ancestor (node). Phylogenetic methods provide the tools to reconstruct the evolutionary history of modules at various organizational levels as a tree diagram, as illustrated by several studies described above.<sup>(20,23)</sup>

### EDB applications for module trees

In the previous section, we demonstrated that modules at various levels of biological organization have an evolutionary pattern that can be reconstructed using phylogenetic methods. In addition to their phylogeny, each level has specific historical associations with other levels as a result of being components of the same organism. Like genes within a genome, these levels can be organized into degrees of hierarchical inclusiveness. However, inclusiveness does not necessarily equate with identical patterns of descent. Comparing phylogenetic patterns between different organizational levels, such as species tree versus gene tree or species tree versus structure tree, identifies discrepancies in the reconstructed evolutionary patterns. Indeed, it is the incongruence between tree patterns across different biological levels that

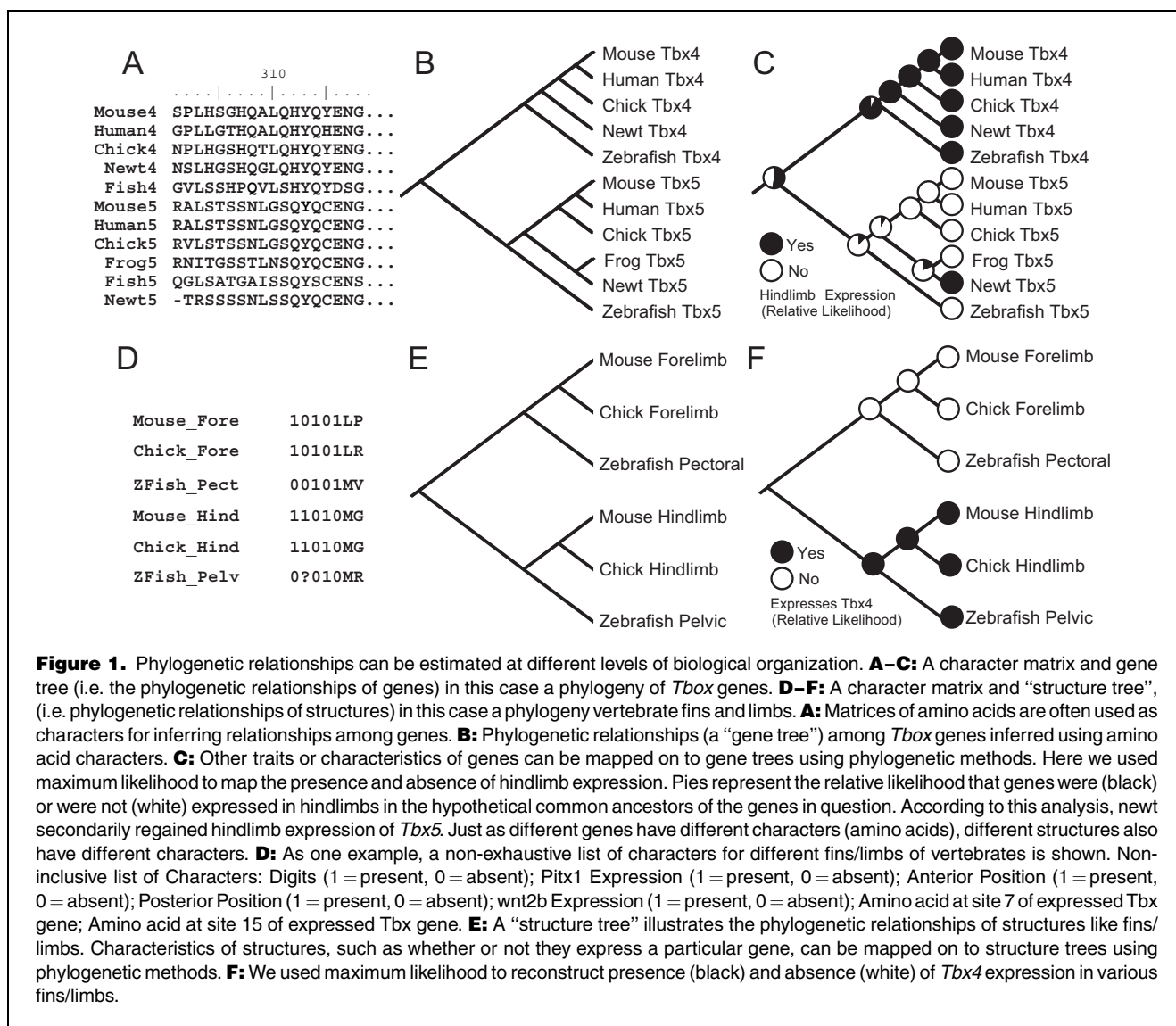
can be used to develop hypotheses of the specific evolutionary processes that created the recovered patterns. Here we describe two existing sets of phylogenetic tools, character mapping and reconciled trees, that compare evolutionary histories between different levels of organization. We argue that these methods can be generalized beyond current common usage to test explicitly many central hypotheses in EDB.

*Character mapping*

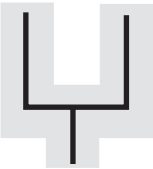
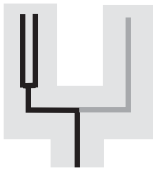
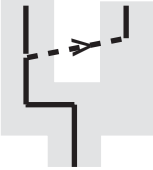

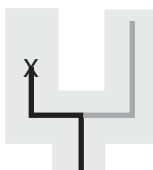
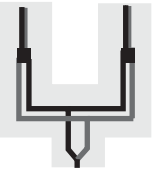
Character mapping is one set of phylogenetic tools that involves comparisons between levels of biological organization. Most commonly, character mapping methods are used to evaluate how traits below the level of species evolve in the context of a phylogenetic tree of species. Consider a phylogenetic tree of beetles. The distribution of characters with

respect to a phylogenetic tree of the beetle species can be used to understand the evolution of particular traits. For example, if two closely related species possess large horns, we would probably hypothesize that they evolved from a common ancestor with large horns. In contrast, distantly related species with large horns may have evolved those horns separately, perhaps through convergent events of sexual selection. The interplay between species' phylogenetic relationships and the character traits of those species can be informative about the order, rate and timing of trait evolution. We maintain that character mapping can be used at many other levels of biological organization besides the common use of mapping characters on species trees.

Just as species have characters (such as horns) that can be mapped on to species trees, modules at other levels of



**Figure 1.** Phylogenetic relationships can be estimated at different levels of biological organization. **A–C:** A character matrix and gene tree (i.e. the phylogenetic relationships of genes) in this case a phylogeny of *Tbox* genes. **D–F:** A character matrix and “structure tree”, (i.e. phylogenetic relationships of structures) in this case a phylogeny vertebrate fins and limbs. **A:** Matrices of amino acids are often used as characters for inferring relationships among genes. **B:** Phylogenetic relationships (a “gene tree”) among *Tbox* genes inferred using amino acid characters. **C:** Other traits or characteristics of genes can be mapped on to gene trees using phylogenetic methods. Here we used maximum likelihood to map the presence and absence of hindlimb expression. Pies represent the relative likelihood that genes were (black) or were not (white) expressed in hindlimbs in the hypothetical common ancestors of the genes in question. According to this analysis, newt secondarily regained hindlimb expression of *Tbx5*. Just as different genes have different characters (amino acids), different structures also have different characters. **D:** As one example, a non-exhaustive list of characters for different fins/limbs of vertebrates is shown. Non-inclusive list of Characters: Digits (1 = present, 0 = absent); Pitx1 Expression (1 = present, 0 = absent); Anterior Position (1 = present, 0 = absent); Posterior Position (1 = present, 0 = absent); wnt2b Expression (1 = present, 0 = absent); Amino acid at site 7 of expressed Tbx gene; Amino acid at site 15 of expressed Tbx gene. **E:** A “structure tree” illustrates the phylogenetic relationships of structures like fins/limbs. Characteristics of structures, such as whether or not they express a particular gene, can be mapped on to structure trees using phylogenetic methods. **F:** We used maximum likelihood to reconstruct presence (black) and absence (white) of *Tbx4* expression in various fins/limbs.

Pattern	Process	Levels of organization (non-inclusive)		
		Species v. gene	Species v. cell	Organ v. cell
	Orthologous duplication	Gene tracks species at time of speciation; species and gene are congruent at that node	Cell type tracks species tree at time of speciation	Cell type tracks organ tree at time of organ diversification
	Paralogous duplication	Gene duplication occurs within a single species lineage	Cell type duplicates within a species	Cell type duplicates and is limited to a specific organ
	Horizontal transfer	Gene or genome is transferred ("jumping") or copied ("duplicative") between species. For example, transposable elements mediated by viruses or lateral gene transfer between unrelated bacterial strains	Cell type is co-opted by a non-sister species lineage. Cell type lineage does not track species tree	Cell type is co-opted by a non-sister organ. Cell type lineage does not track organ tree
	Incomplete tracking	Gene or chromosomal set tracks only one species lineage after speciation event. For example, aneuploidy [monosomy] in plant species	Cell type does not track both lineages after a speciation event; cell type is specific to one lineage. For example, sperm loss in parthenogenetic species	Organs differentiate, but cell type is specific to only one organ lineage
	Loss	Gene is lost (i.e. deleted) in species lineage	Cell type is lost in species lineage. For example, loss of cone photoreceptors in skates	Specific cell type is lost in organ lineage. For example, loss of cone photoreceptors in skate retinas
	Integration	Members of a gene family in a species do not evolve independently, but in a concerted fashion. For example, ribosomal RNA genes in prokaryotic and eukaryotic organisms	Members of a cell family in a species do not evolve independently, but in a concerted fashion	Members of a cell family in an organ do not evolve independently, but in a concerted fashion

**Figure 2.** Phylogenetic patterns and the inferred evolutionary process between different organizational levels, such as species and cell types, species and genes, or cell types and genes. Each pattern has a major, more inclusive lineage (light grey), such as species or cell type, and an ancillary lineage (black), such as gene or cell type. Listed processes are analogous to events that occur between host species and associated parasite lineages. "X"s represent a lineage extinction or loss. Solid squares represent homogenizing mechanisms in concerted evolution or developmental integration.

## Problems and paradigms

organization possess characters that can be mapped on to module trees. As a familiar example, consider genes. Gene trees are ubiquitous in the literature and they are estimated by phylogenetic comparison of their characters, namely nucleotides, amino acids or intron positions. These characters of

genes can be mapped on to the gene tree using phylogenetic tools. Genes also have other characteristics that can be mapped on to gene trees: gene expression is among the most important of these for EDB. Since gene expression patterns can be considered traits of genes, phylogenetic methods can

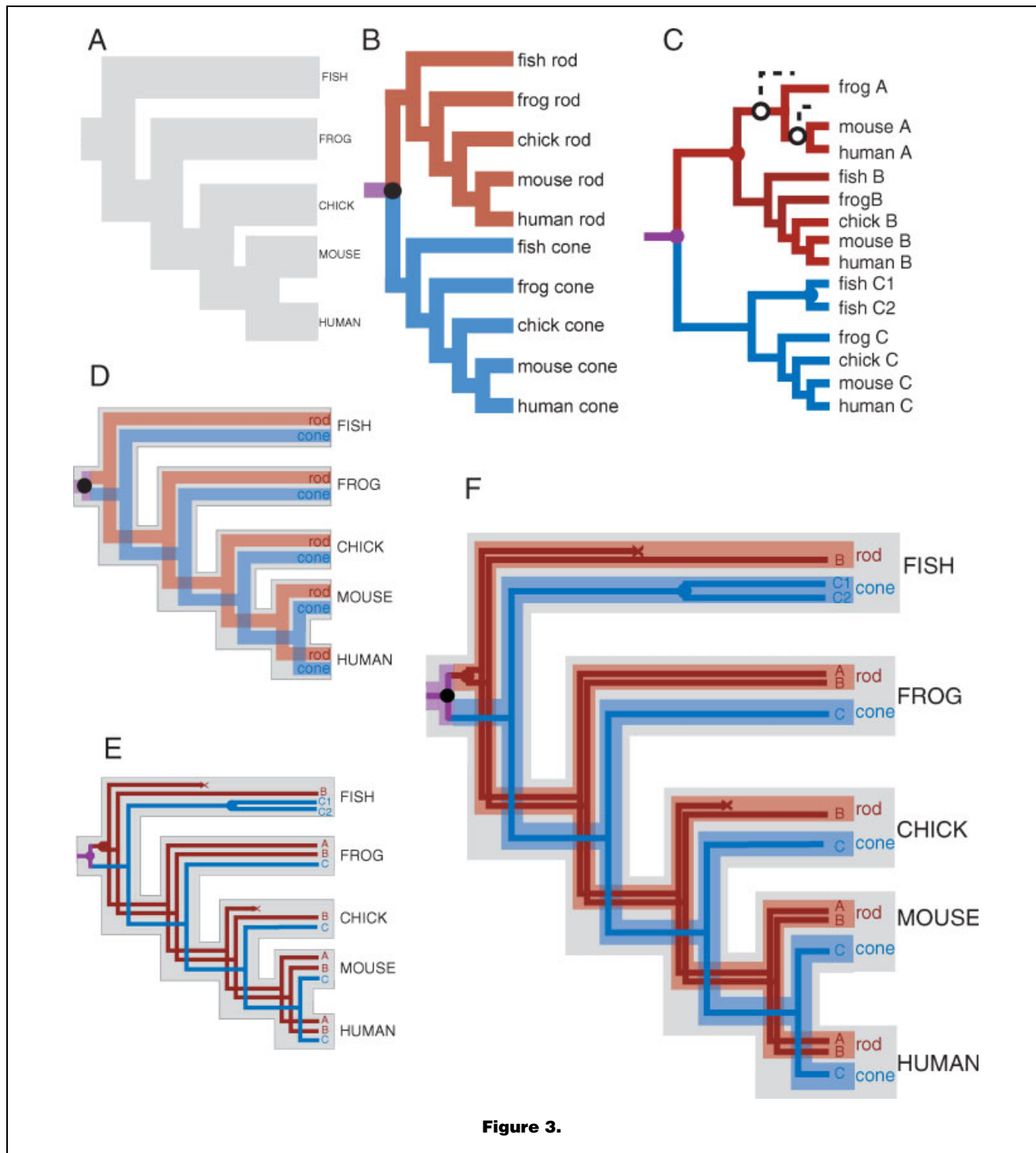


Figure 3.

be used to map expression patterns on to gene trees. For example, “hindlimb expression” is a trait of some *Tbx* genes and it can be mapped on to a gene tree derived from amino acid sequences (Fig. 1C).

Importantly, character mapping can be generalized beyond the more familiar examples of species trees and gene trees. For example, phylogenetic trees may be constructed for morphological structures, such as paired fins/limbs (Fig. 1E). These trees are generated from characters specific to the structures, which may include gene expression patterns, skeletal elements, cell types or developmental fields. These or other characters subsequently can be mapped on to the resulting structure trees, making sure that the unresolved debate about circularity is considered when including mapped traits in the original phylogeny estimation.<sup>(41)</sup> Expression of *Tbx4* is one example of a characteristic of some fins/limbs. This character of gene expression can be mapped on to a phylogeny of the structures themselves (Fig. 1F). Indeed, character mapping can be generally applied to module trees from many different levels of biological organization to examine many kinds of evolutionary hypotheses.

These considerations raise the possibility that the entire toolkit of phylogenetic methods can be extended to modules at multiple levels of biological organization, with many applications for questions in EDB. Detailing all the methods of the character mapping toolkit is beyond the scope of this article and have been reviewed elsewhere.<sup>(42,43)</sup> Nevertheless, a brief overview should be useful as an introduction to the types of hypotheses that can be addressed. Here, we are using the term “character mapping” to refer broadly to phylogenetic comparative methods that include (1) correlative methods, (2) ancestral state reconstruction and (3) rate parameter estimation. All three methods can be used to address questions in EDB.

Correlative methods seek to test hypotheses of correlation between different characters within a particular module/level. Common ancestry causes characters to be non-independent,

violating an assumption of most statistical tests of correlation (like regression). This phylogenetic non-independence can be taken into consideration using methods like independent contrasts<sup>(44)</sup> or analogous discrete-state tests.<sup>(45,46)</sup> Demonstrating evolutionary correlation is important for EDB and phylogenetic methods should be used to account for the non-independence introduced by the common ancestry of species, genes, structures, cell types and other modules. Correlative hypotheses of interest to EDB include changes in gene expression with species/structural/cellular phenotype, changes in specific amino acids with phenotype and changes in promotor sequences with changes in gene expression.

Ancestral state reconstruction methods seek to estimate the state(s) of a character(s) for a common ancestor of a particular group of related modules. These methods have been used to estimate and recreate vocalizations of ancestral frog species<sup>(47)</sup> and amino acid sequences of ancestral proteins.<sup>(48)</sup> An important use of these methods in EDB includes inferring how gene expression patterns evolved. Fig. 1C shows that the ancestor of *Frog-Tbx5* and *Newt-Tbx5* genes was probably not expressed in hindlimbs and hindlimb expression was most likely gained recently in the lineage leading to *Newt-Tbx5*. In this example, gene expression is considered a trait of different *Tbx* genes, but ancestral state reconstruction can also be used at other levels of biological organization. In Fig. 1F, we instead treated gene expression as a trait of a structure module (the fin/limb). This allows the inference (given the presently available data) that *Tbx4* expression is a trait of the structure ancestral to zebrafish pectoral fins and mouse/chicken forelimbs. For illustrative purposes, we presented here simple examples that can be solved by inspection. However, many EDB data sets will require algorithmic estimation of ancestral states using parsimony,<sup>(49)</sup> likelihood<sup>(50)</sup> or Bayesian<sup>(51)</sup> inference techniques.

Rate parameters usually constitute the simple models used by character mapping. Testing hypotheses based on esti-

**Figure 3.** Phylogenetic relationships are estimated from three levels of biological organization (**A–C**) and phylogenetic patterns are compared between the levels (**D–F**). **A:** A species tree of five major vertebrate lineages inferred from morphological and molecular data. **B:** A cell type tree of vertebrate photoreceptors inferred from gene expression data and amino acid sequences. Solid circles represent paralogous gene duplication events. **C:** A gene tree of PDE6 gene homologs (A, B and C) inferred from amino acid sequences. Gene copies are expressed in either rods (red) or cones (blue) photoreceptors. Solid circles represent paralogous gene duplication events, while open circles and dotted lines indicate putative gene deletions or loss of gene expression in that lineage. **D:** Comparison of phylogenetic pattern between species tree (in grey) and cell type tree, where rod photoreceptors are in red and cone photoreceptors are in blue. **E:** Comparison of the species tree (in grey) to the PDE6 gene tree. The gene tree shows evolutionary relationships among PDE6 gene homologs (A, B and C) expressed in either rod (red) or cone (blue) photoreceptors. Solid circles represent paralogous gene duplication events, while “X”s indicate putative gene deletions or loss of gene expression in that lineage. **F:** A possible reconciliation of all three organizational levels. The species tree is in grey; the cell type tree has wide, transparent colored lines; the gene tree is depicted as thin, opaque lines. “X”s represent gene loss. Solid circles represent paralogous duplications between the cell type tree and the species tree (black) or the gene tree and the species tree (colored). Note that a paralogous duplication at one level of comparison (duplication of the PDE6 lineage in an early vertebrate ancestor) can be an orthologous duplication at a different level of comparison (duplication of the PDE6 lineage in the ancestral photoreceptor cell type).

mates of these rate parameters using maximum likelihood<sup>(43)</sup> or Bayesian<sup>(52)</sup> statistical techniques is an important use of character mapping methods. Previous studies have shown, for example, that complex morphology has been more often gained than lost during evolution of fungal species<sup>(53)</sup> and generalists species more commonly evolved from specialists than the reverse.<sup>(54)</sup> By examining modules at multiple levels of biological organization, rate estimates will be important for EDB: Wilkins<sup>(13)</sup> cited rates of developmental evolution as one of three central topics for future work in EDB. For example, we may be interested to learn whether gene expression domains are more commonly lost than gained during evolution, a hypothesis that could be examined by constructing gene trees and mapping gene expression to compare the rate of gain versus loss of expression domains. Similar hypotheses could also be examined at other levels of biological organization.

### *Reconciled trees*

Reconciled tree analysis (RTA) is another phylogenetic tool that makes comparisons between levels of biological organization. The analysis compares and contrasts the patterns of bifurcation between two trees that are historically and hierarchically related (such as species trees and gene trees), allowing inference of evolutionary processes. RTA was originally described<sup>(55–57)</sup> as a generalized analytical approach that can be applied to the diverse disciplines of molecular evolution, parasitology and biogeography. Researchers within these disciplines have used RTA to examine the historical relationship of genes within species, parasites within hosts or species within geographic areas.<sup>(57)</sup> We propose to expand the use of RTA to include lineage comparisons relevant to EDB such as gene trees to cell type trees, cell type trees to structure trees, or structure trees to species trees. We can justify the use of RTA for these finer scale comparisons because the same evolutionary processes occur at multiple levels of biological organization, like paralogous duplication and developmental integration (termed concerted evolution for genes) (Fig. 2). The patterns elucidated by phylogenetics and compared by RTA can help us identify these processes. For example, the patterns of a species tree and a gene tree will not be congruent if a gene lineage in the gene tree was lost (Fig. 2). However, similar incongruence between species tree and gene tree may be observed if a gene copy is horizontally transferred. RTA is a rigorous method to differentiate between evolutionary processes when examining complex data sets from multiple hierarchical levels simultaneously.

One good example of a complex data set where RTA could be applied is the study of Nordström et al.<sup>(58)</sup> on modules of the vertebrate retina. The vertebrate retina is a highly organized neural structure composed of seven major cell types, including two photoreceptor cell types: rods and cones. Many gene families are involved with phototransduction and are ex-

pressed restrictively in specific retinal cell types. Nordström et al.<sup>(58)</sup> phylogenetically examined a suite of these vertebrate gene families and identified multiple duplication events at the cell, chromosome and gene levels that occurred in a vertebrate ancestor. This correlation may suggest causation; evolutionary changes (such as duplication events) at one level may induce or facilitate such changes at other levels.<sup>(38,59,60)</sup> For example, gene duplications resulting from chromosome or genome duplications may have facilitated the differentiation of two retinal cell types, rods and cones, that evolved specific functions, scotopic (dim light) and photopic (bright light) vision, respectively (see Nordström et al.<sup>(58)</sup> for a more detailed discussion).

Although Nordström et al.<sup>(58)</sup> did not use explicit phylogenetic tools to identify correlations when comparing hierarchical levels of biological organization, we advocate the application of methods, such as RTA, to these types of data. For example, we can explicitly examine the historical associations among three hierarchical levels (species, cell type and gene) in the vertebrate retina by generating phylogenetic trees for each, then comparing their historical patterns with RTA. Our species tree followed the well-supported phylogeny of the major vertebrate lineages (see review in Meyer and Zardoya<sup>(61)</sup>) (Fig. 3A). The cell type tree was constructed from gene expression data (compiled by Arendt<sup>(19)</sup>) and amino acid sequence changes of opsin homologs expressed in specific cell types (Fig. 3B). The gene tree of PDE6 homologs was generated from amino acid sequences across five species representing major vertebrate lineages: pufferfish, frog, chicken, mouse and human (from Nordström et al.<sup>(58)</sup>). We then compared two levels of historic associations at a time (vertebrate species versus retinal cell type, species versus PDE6 gene, retinal cell type versus PDE6 gene) using a reconciled tree illustration (*sensu* Page<sup>(62)</sup>) (Fig. 3D, E). Finally, we depict one possible reconciled tree including all three levels of biological organization (Fig. 3F).

Our results using RTA reflect the conclusions of Nordström et al.,<sup>(58)</sup> where there is an association of duplication events at two different levels of organization—genes and photoreceptor cell types—in a vertebrate ancestor. The timing of these events suggest that change at the gene level may have facilitated a duplication and subsequent diversification, at level of the cell type resulting in the origin of two types of vertebrate photoreceptors, rods and cones, and two functions, scotopic and photopic vision. Unlike Nordström et al.,<sup>(58)</sup> our approach uses explicit phylogenetic methods for a rigorous comparison between hierarchical levels so that the order and timing of trait evolution can be assessed quantitatively.

Although, our simple example of a reconciled tree (Fig. 3F) can be solved by inspection quite easily, many data sets are more complex. For example, the number of gene copies in a gene tree may not match the number of taxa in a species tree or the pattern of the gene tree may have little or no similarity

to the species tree pattern. We argue that RTA is an objective tool that can be applied to complex data sets to develop evolutionary hypotheses.

### Conclusions

Many central topics in evolutionary developmental biology involve the comparison of evolutionary histories of modules from different levels of biological organization.<sup>(1,14,63)</sup> This hierarchical and phylogenetic view of evolutionary developmental biology is not completely new: phylogenetics has been routinely applied to hierarchical questions in EDB.<sup>(14)</sup> However, previous phylogenies have been constructed only for species and genes, representing only two of many possible levels of biological organization. In fact, usually only one level—species trees—has been used for mapping developmental characters. Here we point out that researchers have begun to construct phylogenetic trees from units (modules) of other levels of organization, indicating that the methods can be generalized to bifurcating modules from any level. Several phylogenies already have been constructed at the level of structure or organ: Coates et al.<sup>(24)</sup> estimated a tree of tetrapod appendages, Geeta<sup>(20)</sup> estimated a tree of leaf primordia and Oakley<sup>(21)</sup> and Liu and Friedrich<sup>(22)</sup> illustrated the concept of structure trees for photoreceptors. In addition to structure-level trees, Arendt<sup>(19)</sup> conceptualized phylogenetic trees of retinal cell types and Cunchillos and Lecointre<sup>(23)</sup> explicitly estimated phylogenetic trees of metabolic pathways. These separate articles provide examples for module trees of structures, cell-types, and metabolic pathways, in addition to the common trees of species and genes. These examples already represent module trees from five different levels of biological organization and point to the generalization of phylogenetic methods whereby trees can be constructed for modules at any level of biological organization using traits of those modules. The generalized hierarchical phylogenetics advocated here indicates the exciting possibility of co-opting the entire phylogenetic toolkit to address fundamental questions in EDB.

The interest in phylogenies comes not from building them, rather from what they tell us about evolution.<sup>(64)</sup> Just as species trees inform us about the evolution of species' characteristics, module trees can inform us about the evolution of modules' characteristics, which is perhaps the fundamental goal of EDB. We have pointed out that module evolution can be investigated using two suites of phylogenetic tools, character mapping and reconciled trees. We use "character mapping" to refer to phylogenetic comparative methods, including correlational methods, ancestral state reconstruction, and rate parameter estimation. Applying these methods to new levels of biological organization will allow testing of central hypotheses in EDB, such as correlations between particular genotypic and phenotypic changes during evolution, tempo and mode of gene expression<sup>(7)</sup> and developmental pathway evolution, co-option, and developmental integration. Similarly,

reconciled trees—when generalized to new levels of biological organization—will provide new insights into EDB by revealing processes of evolution that span levels of organization. These processes include orthologous and paralogous duplication (of for example, cell types and structures), horizontal transfer, and loss (Fig. 2). Often, the timing of such evolutionary events may suggest the primary origin of evolutionary novelty, such as when gene duplications precede cell type or organ differentiation.

Evolutionary developmental biology aims to understand the history of hierarchical and modular systems. Phylogenetics is an established system of methods for dealing with the history of hierarchical systems. We maintain that phylogenetics is currently under utilized because it primarily focuses only on species and gene levels. Generalizing phylogenetics to bifurcating modules at all levels of biological organization can provide a valuable analytical framework for testing fundamental EDB hypotheses using well-established, versatile and sophisticated statistical methodologies.

### Acknowledgments

We thank E. Abouheif, J. Endler, R. Geeta, J. Jeffery, R. Mayden, K. Nordström, S. Parker, K. Roe, members of the Oakley lab group, A. Wilkins and two reviewers for valuable comments that greatly improved this article.

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