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The place of phylogeny and cladistics in *Evo-Devo* research

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Introduction

Understanding the evolution of the great diversity of animals is a major goal of biology. We would like to understand how evolution has happened in an historical sense - which characteristics arose in which lineages, when they arose and even offer adaptive explanations of why they arose. More generally we would like to go beyond the neodarwinian explanation of adaptation through selection on random mutations to discover exactly what kind of changes at the level of the genotype have given rise to the changes we see in phenotype.

We can summarise these intellectual goals as a series of different entities of interest to an evolutionary biologist. First is knowledge of the evolutionary relationships of taxa as represented by a phylogenetic tree. Next, consideration of the distribution on this phylogeny of different character states (genotypes or phenotypes) possessed by the different species can lead to various possible forms of interpretation: we can make inferences about the homology of characters because identical characters in adjacent branches are likely to be homologous, we can reconstruct the common ancestor of two taxa as any characters the extant taxa share must have been present in and inherited from their common ancestor and we can consider the changes in character states between ancestral nodes on a tree to reconstruct the historical path of evolution. Finally we can make inferences about the mechanism of evolution that has resulted in the different character states distributed on the phylogeny. When reading this manuscript it will become strikingly clear that all of these aspects of cladistics have a complex, interdependent relationship all ultimately based on the criterion of parsimony. To make our discussion as clear as possible we have employed the following structure:

1. Cladistic tree reconstruction

2. Determination of character homology
   - *Argument from parsimonious distribution on a tree*
   - *Using molecular trees*
   - *A digression on character optimisation*
   - *Argument from complexity*

3. Reconstruction of ancestors
   - *The new animal phylogeny: Ecdysozoa, Lophotrochozoa, Deuterostomia*
   - *Evidence for and against the new animal phylogeny*
   - *Homology of arthropod and vertebrate segmentation and its presence in Urbilateria*
   - *Broadening the definition of segmentation*
   - *Genes and the homology of segmentation in arthropods and chordates*

4. History of character evolution
   - *Tracing character evolution*
   - *Gene phylogenies*
   - *Character polarisation*

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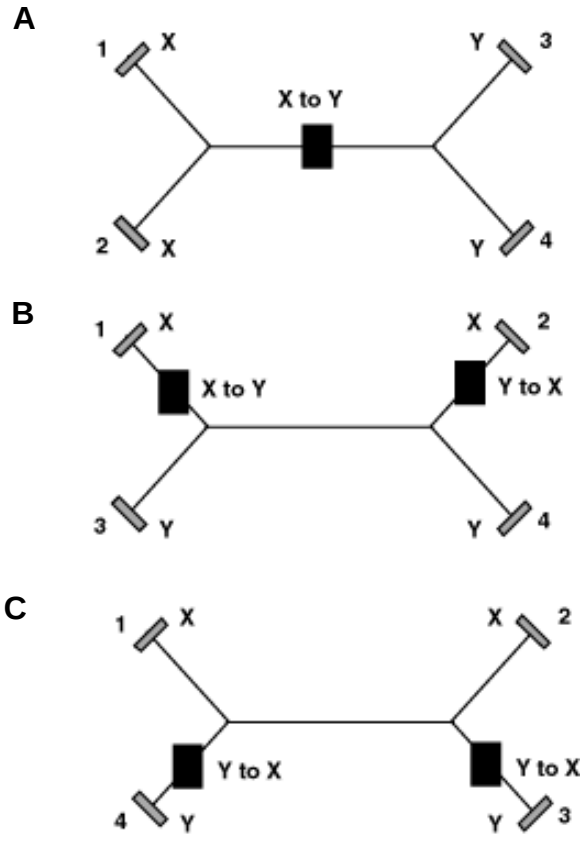


Fig. 1. The principle of cladistic analysis. *Unrooted trees relating four taxa, showing the three possible outcomes in A, B, and C. The numbered grey bars indicate the character states of terminal taxa either X or Y. In tree* **(A)***, a single change from X to Y can account for the distribution of characters. In* **(B)** *and* **(C)***, two changes are needed to account for the distribution of characters. Parsimony analysis would select scenario A as the tree of choice. If we know that taxon 1 is the outgroup then the derived character Y and taxa 3 and 4 is a synapomorphy.*

5. Explaining evolution
- *The comparative method*
- *Linking genotypic change to phenotypic change*

1. Cladistic tree reconstruction

Whilst the study of systematics originated in an explicitly non-evolutionary context, it became explictly applied to evolutionary questions by the end of the nineteenth century, above all by Haeckel e.g. (Haeckel 1866). Naïvely, it was thought that patterns of relationship automatically gave an insight into lines of descent, above all when combined with a literal reading of, for example, the fossil record, or early embryology. This approach was found to give diminishing returns, and something of a crisis in the subject had emerged by the 1970's.

Modern practice relies on phylogenetic systematics, growing out of the seminal work of Willi Hennig (e.g. Hennig, 1966). Hennig's great achievement was to undermine the use of primitive shared characters (and often, therefore, overall similarity) as the basis for systematics.

Here we deal very briefly with the Hennigian, cladistic approach to reconstruction of phylogenetic trees; for a much fuller discussion see e.g. (Kitching *et al.,* 1998). We concentrate on morphological characters but it is important to make clear that exactly the same considerations can be applied to molecular data. In Hennigian systematics, the procedure begins with a number of characters that are scored for their state or, more often in the case of morphological characters, their presence or absence, in each of the taxa of interest. At this stage there is an *a priori* assumption of homology of characters that are coded the same in different taxa – this as yet unproven belief in homology is termed primary homology. Using the criterion of parsimony we select as best supported the tree amongst all possible trees (over 2 million possible rooted trees for 10 taxa) that minimizes the implied number of changes in these characters over the tree (Fig. 1). The characters that change at the bases of derived clades are referred to as synapomorphies, as opposed to those inherited from more distant ancestors, or plesiomorphies (Figs. 1,2). It is consideration of these synapomorphies that we will consider in detail in the next section.

2. Determination of character homology

One of the most important uses to which phylogenies are put in cladistic analyses of morphology is determination of homology (true similarity owing to common descent) versus homoplasy (convergent similarity). Indeed, purists would argue that the ultimate test of homology is its identification with synapomorphy: i.e. the term "homologous" should only be applied to synapomorphic character states on trees. The importance of homology determination is clear: we have discussed the primary assessment of homology as a prerequisite for cladistic tree reconstruction, and we will see the

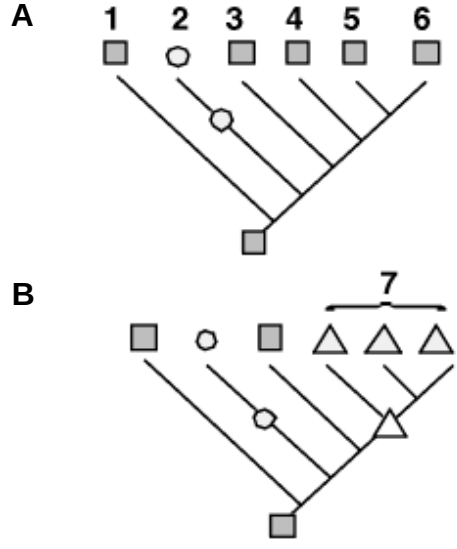


Fig. 2. Primitive and derived characters. *In tree* **(A)***, taxa 3-6 are characterised by the same character state. However, it would be wrong to infer from this alone that they formed a clade, as the presence of the same character in 1 suggests this character is plesiomorphic (i.e. primitive) for the clade in question, and therefore does not indicate particular relationship. In tree* **(B)***, the triangle character state is a synapomorphy (i.e. a shared derived character) of clade 7, and therefore correctly marks a true clade.*

central place of homology for reconstruction of ancestors and all that this leads to. Conversely, homoplasiously evolved characters are the bread and butter of the comparative method that aims to tell us why the different charactersistics of organisms have evolved. We also consider in this section the problem of character optimisation: essentially how evolutionary inferences are drawn from the character distribution of the organisms in a tree.

Argument from parsimonious distribution on a tree

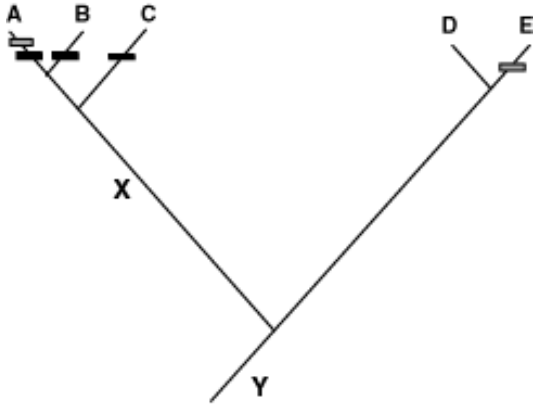
As we have explained, the cladistic procedure begins with a matrix of characters in which there is a statement of belief about the homology of these characters. This is a statement of belief in the *primary* homology of the characters and this needs to be tested within the context of a cladogram. Truly homologous characters will be found to be adjacent on a cladogram (which is constructed using all of the characters) such that their appearance can be accounted for by a single evolutionary event (Fig. 3).

Of equal interest to such identified synapomorphies will be the characters whose primary homology is not corroborated. As an example, the hemichordates and the lophophorates (e.g. brachiopods) have strikingly similar, ciliated feeding structures called lophophores (e.g. see (Nielsen 1987)). Based on the co-occurrence of lophophores as well as other embryological shared characters, the brachiopods and relatives were long thought to be close relatives of the deuterostomes (including the hemichordates), but recent molecular phylogenies show the two clades are not closely related suggesting that, incredibly, these beautiful and complex structures have been evolved convergently in the two clades. Demonstrating homology or homoplasy in this way is rarely this straight-forward, one example is given in Fig. 4 where there are two equally parsimonious reconstructions of the evolution of a character. In this example, one of the reconstructions implies a single gain (i.e. that the character is homologous in the 2 taxa that possess it) and a single loss. The second reconstruction assumes no losses but two gains meaning the characters are convergently evolved and not homoplasious.

Using molecular trees

Homology determination is one of many areas in which molecular analyses may provide independent assessment of homology claims. First, molecules are providing independent trees on which to plot the distribution of characters, breaking the link between tree building and homology assessment described above. Later we will see that molecules are providing evidence directly supporting putative homologies most notably through the analysis of the expression of homologous genes in the structures of interest.

The first contribution of molecular biology has already been exemplified by our consideration of the lophophore whose distribution on the *molecular* tree of metazoans revealed it to be convergently evolved in the hemichordates and the brachiopods. Another intriguing illustration is the argument over the homology or otherwise of the halteres (gyroscopic balancing organs) derived from the posterior



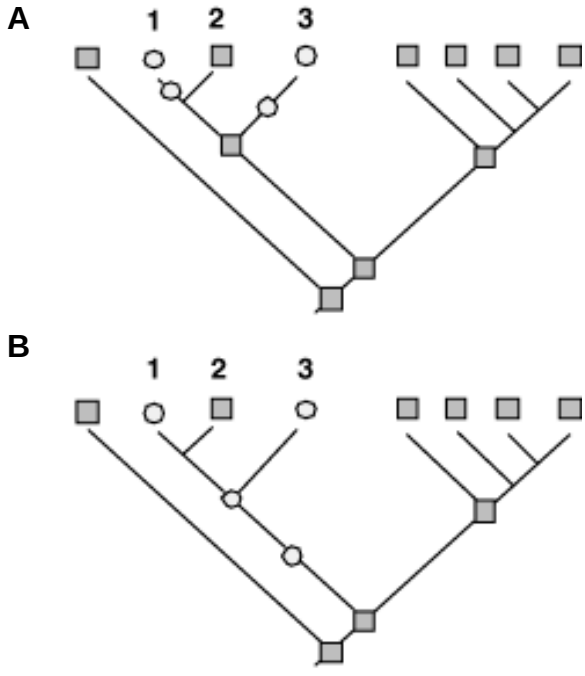


Fig. 3 (Left). Tree-based homology: homology as synapomorphy. *Two sets of putatively homologous characters, represented by the grey and black bars, have been mapped on to a parsimonious tree. Based on their distribution, it may be seen that the most parsimonious interpretation of the black character states is that all the occurrences are homologous and were acquired as a synapomorphy of the clade they occur in, at point X. The grey character states, conversely, do not show such an adjacent distribution and must be considered to be convergences (requiring their appearance on two occasions. If the grey bars are to be considered homologous they must have appeared once at position Y and subsequently been lost three times in the lineages leading to B, C and to D making a less parsimonious total of four changes. The tree thus supports the homology of the black character states, but not of that of the grey ones.*

Fig. 4 (Right). Character optimization problems I. *The two trees given in A and B are identical in terms of character assignment to terminal taxa and topology. However, reconstruction of character states at internal nodes differ according to whether evolutionary change takes place early or late. In* **(A)** *change is delayed, so that the character states of taxa 1 and 3 are independently derived. In* **(B)**, *change takes place early, so that the entire clade of 1+2+3 is characterised by the circle character state, and taxon 2 has re-evolved the square state; a reversal. Both options are equally parsimonious.*

pair of wings in the dipteran insects and from the anterior pair of wings in the strepsipterans. The structures themselves are strikingly similar apart from being in the wrong place. The reason that this possibility is so important is that if they are shown to be homologous then this would imply an extraordinary homeotic transformation of third thoracic segment to second and vice versa in the strepsipterans (Whiting and Wheeler 1994)! The argument over the homology of these halteres has been based on their distribution on a tree of the insects. If the strepsipterans can be shown to be tightly linked to the dipterans then homology of their halteres will be supported. If flies and strepsipterans are separated on the tree by taxa with wings rather than halteres then convergent evolution of halteres is the preferred explanation. This aspect of insect phylogeny remains unresolved although it seems the balance of evidence supports independent haltere evolution in the two clades (Whiting and Wheeler 1994; Rokas *et al.,* 1999).

A digression on character optimisation

Using the straightforward parsimony approach described, it is normal not to constrain the phylogenetic cost of any particular transformation. Whilst this minimises the number of potentially unwarranted assumptions about the evolutionary process, it can lead to rather disturbing conclusions. One typical outcome is that intuitively trivial changes may be accorded equal significance to profound reorganisations: in a sense this problem equates to the probability that such a profound reorganisation actually consists of lots of smaller changes. Furthermore, the directionality of change may also be thought to have different evolutionary cost (Fig. 5). Such intuitions have been concretised into the idea of Dollo parsimony, which in essence assumes that it is easier to lose a complex character than it was to evolve it in the first place hence, a scenario of character evolution that assumes multiple convergent gains of a character ought to be penalised more heavily than an alternative scenario

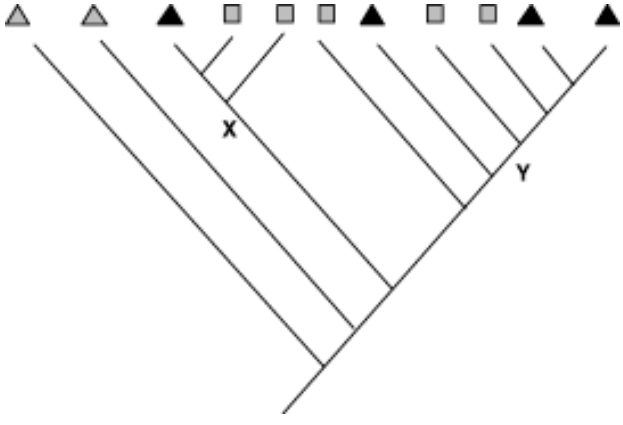


Fig. 5. Character optimization problems II. *Reconstructing evolution of a character when gain and loss are not equally plausible. In the tree shown, the triangle character state is apparently present at the base. However, the square states represent taxa that do not possess this state. If a strict, equally-weighted parsimony criterion is applied, then the black triangles would represent re-appearance of the triangle character state, after its loss at X and Y. If, however, the loss of the triangle state is weighted such that its loss is more likely than its re-appearance, then all the triangular character states could be true homologies, with separate losses of the state occurring in each of the branches characterised by the square state. Typical examples might be the loss/re-evolution of wings in insects or the loss/re-evolution of the coelom in bilaterians.*

which assumes a unique evolution then multiple losses of this character. (This is a (deliberate) misinterpretation of Dollo's law (Dollo 1893) which actually states that it is generally impossible to revert back"*precisely* to an ancestral character state). Part of the difficulty lies in the need to decide *a priori* how likely it is that a character could be convergently gained or secondarily lost. This in effect involves an *a priori* estimate of the probability of homology of the derived state in the different taxa.

One much-discussed example is that of insect wings. Goloboff points out (Goloboff 1997) that if one had a winged arthropod in front of one, it would certainly be an insect; but that if one had a wingless arthropod, it does not need to be a non-insect: wings are known to have been lost on many occasions in insects. This example suggests that loss is indeed easier than gain.

However, a recent study on stick insects arrives at the opposite conclusion (Whiting *et al.,* 2003). Whiting *et al.,* have looked at the presence and absence of wings across a molecular phylogenetic tree of the stick insects. They show that six independent clades at the base of the stick insects lack wings while four or five groups nested within these are winged. They claim that the most likely explanation of the observed pattern of presence and absence of wings is that wings were lost once in the ancestor of the stick insects and have been re-evolved as many as four times. The alternative interpretation requires 13 independent occasions of wing loss.

There are two principal problems with the interpretation of their tree. The first is that we, along with Goloboff, expect *a priori* that wings are more easily lost than gained, and indeed have empirical support for this view ("flight loss has occurred in nearly all winged orders of insects, many times within most orders and probably thousands of times within the Coleoptera" (Wagner and Liebherr 1992)); so the less parsimonious reconstruction might just be the correct one. Second, although it is true that most genes have multiple roles that would tend to preserve their coding sequences, their correct functioning in a specific process such as wing patterning tends to rely on regulatory elements dedicated to that process. In an ancestral wingless stick insect there would be no selection acting to preserve these putative wing specific enhancers over 250 million years in each and every one of the genes that are involved in patterning an insect wing.

Argument from complexity

The tree based approach determining homology described above is one of the most obvious differences to the approach most molecular biologists would instinctively take and this is because homology tends to be more easily directly demonstrable in many molecular characters compared to morphological characters. If we consider the 18S rRNA genes of two animals, we have little doubt that they are homologous because the similarities are extensive and are hugely unlikely to be accounted for by chance or convergent similarity. The tree-based approach still has a place in such considerations of molecular homology due to the existence of gene duplications. The 18S gene from one species is paralogous rather than orthologous to the mitochondrial 16S gene from another and a tree-based comparison is probably the best way to reveal this relationship between the two genes.

The idea that we might put more emphasis on the likelihood of homology of some characters with particularly complex similarities than on those sharing more simple ones leads to a consideration of tree-independent assessment of homology. This would use the criterion of complexity in much the same way that we do when

noticing the extensive and detailed similarities of the nucleotide or amino acid sequences of two homologous genes. The argument essentially is that the more subtle and numerous the similarities between two characters, the more likely it is that they are homologous. The wings of a bee and a dragonfly are similar in their intricate pattern of venation, their two-layered construction, the many different muscles that move them, their position on the 2nd and 3rd thoracic segments etc. etc. They have none of these in common with the wings of birds.

An important, but controversial development in evolution of development research has been the use of molecules, especially developmental genes actually involved in the specification of morphological structures, to aid in this direct assessment of character homology. The broad approach is as follows: if two organisms both possess a similar structure, and the developmental basis for the structures appears to be the same, the implication is that their last common ancestor also possessed such a structure and manner of development. The genes involved in the patterning of bee and dragonfly wings are an additional level of complexity. It is relatively easy to determine that two genes are homologous, the coincident involvement of such an homologous gene in a putatively homologous structure is a persuasive supplementary layer of complexity adding to the case for homology.

Such an argument exalts the complexity criterion over the tree-based criterion for homology to an extreme degree, and certainly calls for some caution. For a start, the same developmental systems are also used to regulate structures that no-one would think of as being homologous (e.g. distalless in both neural crest, and limbs (Nielsen and Martinez 2003)). The use of complexity in determining the homology of characters, in particular the coincident involvement of homologous genes, will be returned to later when we come to consider the characteristics of the ancestor of the Bilateria.

3. Reconstruction of ancestors

If we are able to determine homology of a given character in two taxa in the manner previously described, then it also follows that this character was present in the common ancestor of the two taxa. Great attention has recently been focused on the significance of the shared features of the model organisms such as mouse, fly and worm because the more distant these organisms are from each other, the deeper in bilaterian phylogeny their shared characters evolved. In the light of the 'new' animal phylogeny (Adoutte *et al.,* 2000) flies and worms appear to be very deeply divided from chordates such that their shared characteristics can be inferred to have existed in the common ancestor of all bilaterian animals – an elusive beast called "Urbilateria" (De Robertis and Sasal 1996; Erwin and Davidson 2002). We begin with a discussion of the new animal phylogeny and in particular the contradictory evidence for the profound division between flies and vertebrates. This leads us on to a consideration of the possible character of Urbilateria.

The new animal phylogeny: Ecdysozoa, Lophotrochozoa, Deuterostomia

The most widely accepted current model of bilaterian relationships based on molecular evidence is summarised in Fig. 6 (Aguinaldo *et al.,* 1997; de Rosa *et al.,* 1999). This model has a fundamental split between deuterostomes on one side (chordates plus echinoderms and hemichordates) and protostomes on the other. Within the protostomes there is a further split between ecdysozoans (creatures that undergo moulting or ecdysis - namely arthropods, nematodes, nematomorphs, priapulids, loriciferans and kinorhynchs) on the one hand and the somewhat awkwardly named lophotrochozoans which is a grouping of animals with lophophores (brachiopods, phoronids and ectoprocts) and animals with trochophore larvae or similar (annelids, molluscs, platyhelminths, echiurans, sipunculids, nemerteans) as well as a few that have neither (e.g. rotifers+acanthocephalans, cycliophorans, gastrotrichs). Basal to all these bilaterian phyla (and hence outside of the reconstructions based on arthropod/chordate comparisons) are the acoelomorph worms (Ruiz Trillo *et al.,* 1999; Ruiz-Trillo *et al.,* 2002; Telford *et al.,* 2003). This scheme of relationships is in contrast to the scheme implied in most textbooks of zoology e.g. (Brusca and Brusca 1990) of evolution from basally branching acoelomate platyhelminthes via an intermediate grade of pseudocoelomate worms such as nematodes to a monophyletic group of coelomate animals – arthropods, annelids, lophophorates and deuterostomes. To emphasise the relevance of phylogeny to ancestor reconstruction, it is worth noting that under this textbook phylogeny, characters shared by arthropods and chordates could be extrapolated back only to the common ancestor of the Coelomates (Urcoelomata?) and not to Urbilateria. The other point worth emphasising here is the close alliance of segmented annelids and segmented arthropods (the Articulata) according to pre-ecdysozoan theories in contrast to their separation under the Ecdysozoa hypothesis.

Evidence for and against the new animal phylogeny

Arguably the best evidence concerning the phylogeny of the Metazoa comes from sequences of the small and (latterly) the large subunit ribosomal RNA genes (SSU and LSU) (Aguinaldo *et al.,* 1997; Mallatt and Winchell 2002). Although the rRNA data sets undoubtedly have shortcomings (and their inability to resolve relationships within the Lophotrochozoa makes sceptics of some) these are made up for, at least to some extent, by the size of the datasets (many hundreds of metazoan sequences) and broad taxonomic coverage. An analysis of SSU specifically avoiding taxa with accelerated substitution rates was the first to support the Ecdysozoa clade (Aguinaldo *et al.,* 1997) and subsequent analyses

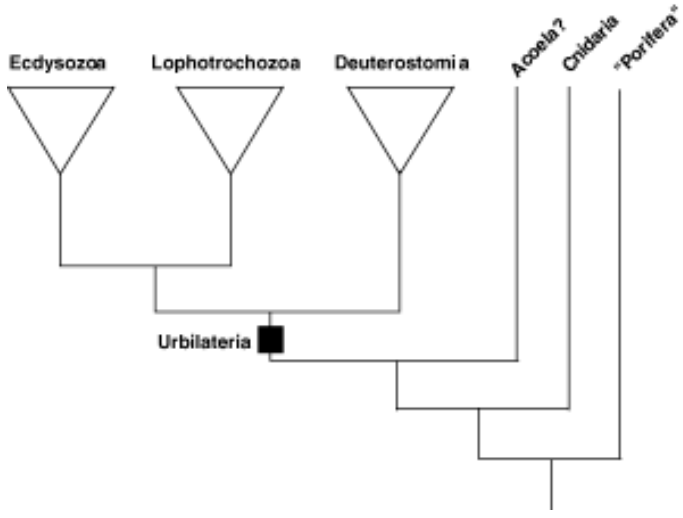


Fig. 6. A schematic verison of the «new» metazoan phylogeny based on molecular data. *Homologous characters shared by arthropods (within the Ecdysozoa) and vertebrates (within Deuterostomia) must have been present in their common ancestor right at the base of the Bilateria in Urbilateria. See text for references and details.*

with different taxonomic composition and supported by LSU studies consistently recover both the protostome/deuterostome dichotomy and, within the protostomes, the Ecdysozoa/Lophotrochozoa split (Mallatt and Winchell 2002). We now consider further evidence both for and against this "new animal phylogeny" that has been provided by other molecular studies.

Hox genes have a highly conserved, 60 amino acid homeodomain with, typically, 5 or 6 amino acids on either side that can be reliably compared between paralogous genes and between phyla. On the face of it, there is little to recommend them for use as phylogenetic markers. What has happily made them fit for this purpose is a characteristic shared with the rRNA genes: the Hox genes have been sampled from a wide diversity of taxa with the result that what phylogenetic information there is within them is clearly understood. Although a complement of as many as 8 Hox genes seem likely to be a primitive characteristic of the Bilateria, various authors have shown that there are characteristics of individual Hox genes that are phylogenetically informative. In particular the work of Balavoine and co-workers has shown the presence of a conserved peptide at the C-terminus of the homeobox of the Lox-5 gene of various trochophoran phyla is found also in the lophophorate brachiopods as well as in the acoelomate flatworms in support of the Lophotrochozoa (Balavoine 1998; de Rosa *et al.,* 1999; Balavoine *et al.,* 2002). The linking of ecdysozoans and lophotrochozoans as protostomes is supported by the common occurrence of genes with the UbdA peptide and the deuterostome, lophotrochozoan and ecdysozoan groupings are each supported by other clade specific posterior class genes (de Rosa *et al.,* 1999). The only potential problem with so-called signature peptides such as Lox-5 is with character polarisation. Because there is no direct knowledge of the ancestral character state (we cannot sample Urbilateria) the presence of a lox-5 peptide might be derived and supportive of a lophotrochozoan clade or it might be an uninformative primitive character. Telford (2000) has pointed out the usefulness of the outgroup relationship between different Hox orthologs (Hox genes are presumed to have arisen through duplication) which can act as proxy outgroups to each other. Following this procedure it has been possible to polarise these characters (Telford 2000a).

Support for the ecdysozoan clade has been claimed from two further molecular studies. Examination of the *Drosophila* and *Caenorhabditis* genome sequences has revealed a triplicated β-thymosin gene in both fly and nematode (Manuel *et al.,* 2000). In other taxa the protein product of this gene was said to consist of a monomeric thymosin protein. However, searches of more recent genome data and EST databases show that the multimeric form is not in fact restricted to the Ecdyoszoa (Telford, in press): in addition to the monomeric molecule, the multimer is found in lophotrochozoans (the platyhelminth *Schistosoma* and the gastropod mollusc *Hermissenda crassicornis*) and in Deuterostomes (*Ciona intestinalis*) and even in fungi and so it is a primitive character that provides no support for the Ecdysozoan clade. The apparent absence of the single copy form from nematodes and dipterans may, however, be seen as giving some support to Ecdysozoa although the significance of the lack of this character is less clear.

The second molecular synapomorphy supporting Ecdysozoa is the presence, in the nervous system of all sampled ecdysozoan taxa, of an antigen recognised by an antibody against Horse-Radish Peroxidase (Haase *et al.,* 2001). This antigen has been partially characterised in *Drosophila* but its genetic basis even in this species is far from clear. The antibody recognises carbohydrate moieties attached to a number of proteins in the cell membrane of nerve cells. The major protein to which these carbohydrates are attached, NERVANA (Sun and Salavaterra 1995) is not restricted to the Ecdysozoa and so the presumed Ecdysozoa specific character must be either a novel biosynthetic pathway leading to the synthesis of these carbohydrates or a pathway directing their attachment specifically to these proteins in the nervous system. The two mutants affecting the presence of the HRP antigen in *Drosophila* offer some clues: one, *Tollo*, has recently been characterised and shown to code for a Toll-like receptor and is expressed in cells adjacent to those possessing the antigens (Seppo *et al.,* 2003). *Tollo* directs the presence of the HRP antigen in a subset of the nerve cells. However, Toll-like genes are not specific to the Ecdysozoa either and *Tollo* appears to have paralogs but no direct ortholog in *C.elegans* (MJT unpublished observations). The other *Drosophila* mutant that affects HRP antigen production, NAC (Haase *et al.,* 2001), is less well characterised being part of a deletion affecting 31 predicted genes. In short, the genetic basis of this HRP antigen remains out of reach, as does the direct demonstration of homology between different ecdysozoan taxa yet this is an intriguing character adding some support for the ecdysozoan clade.

Not all molecular phylogenetic studies support the division of the protostomes into Ecdysozoa and Lophotrochozoa. The shared presence of an unusual fused pair of tRNA synthetase genes in *Drosophila* and vertebrates compared to their separate nature in nematodes and outgroup taxa has been remarked previously (Telford 2002). Additionally, several studies of multiple protein coding genes culled from model genome sequences have all found greater support for linking flies and vertebrates in a coelomate clade rather than linking the flies with nematodes as required by Ecdysozoa (Mushegian *et al.,* 1998; Blair *et al.,* 2002). The most recent of these studies concatenates one hundred genes from flies, nematodes, vertebrates and outgroups and finds overwhelming evidence for Coelomata rather than for Ecdysozoa(Blair *et al.,* 2002). On the face of it the evidence from one hundred genes should count for more than the results from a single gene: SSU. However, the SSU dataset has the great advantage that it has been sequenced from many hundreds of metazoans. It was the ability to select amongst these many sequences for those from taxa (especially nematodes) that did not have significantly elevated rates of substitution that enabled Aguinaldo *et al.,* to identify the ecdysozoan clade in the first place. This selection of more slowly evolving sequences was not possible for the majority of the sequences in the multiple gene data sets reducing their value considerably.

Despite some evidence to the contrary and uncertainties over the strength of the beta-thymosin and HRP studies then, it is our opinion that the balance of evidence supports the Ecdysozoa hypothesis.

We are now in a better position to take up the problems of homology of characters across the Bilateria. It should be noted that, on the face of it, the new phylogeny gives less support than the old to traditional views of homology such as that of segmentation (annelids and arthropods are not sistergroups) and the coelom (coelomate taxa are interspersed with acoelomates and pseudocoelomates) (Adoutte *et al.,* 2000). However, paradoxically, the continued elucidation of shared molecular developmental characteristics has led to increased support for just such homologies! In other words, the use of shared developmental genetics as an argument for complex similarity and thus homology discussed above has been applied in a more aggressive manner to attempt to demonstrate the presence of morphology in deep ancestors (Slack

et al., 1993; Arendt and Nübler-Jung 1999; Arendt *et al.,* 2001; Arendt and Wittbrodt 2001). Here, identification of complex shared developmental systems in any pair of organisms is used as an argument for their presence in the last common ancestor, *and thus for the presence of the structures they now regulate.* Given that bilaterians seem to share the molecular basis for development of structures as diverse as eyes, segments and limbs, the implication would be that the last common ancestor of them all possessed all of these structures. How can this dilemma be resolved? In some cases, the answer is straightforward. For example, the flatworms have a relatively simple body plan, most obviously lacking a through gut with separate mouth and anus (see Fig. 6). Their simplicity was long thought to be a primitive trait of a basally branching bilaterian group. Several recent strands of molecular evidence show that flatworms are not in fact basal worms (apart, perhaps, from the Acoelomorpha). The majority of flatworms form a monophyletic group within the Lophotrochozoa: one of the two major protostome superphyla e.g. (Balavoine 1998). A complete through gut is present in all of the other branches of the Bilateria and so is likely to be a primitive character of Urbilateria. The obvious inference is that, amongst other characters, the flatworms have secondarily lost their through-gut. This new interpretation is not too costly in terms of evolution – a single character change at the base of the platyhelminths - though the loss of such a useful feature does seem surprising.

Homology of arthropod and vertebrate segmentation and its presence in Urbilateria

A much more problematic example is provided by segmentation. The segmentation seen in both annelids and arthropods was long presumed to be a homologous character and was the central feature of the Articulata hypothesis that linked these two clades. On the other hand, the segmentation of arthropods and of vertebrates has been thought to be a case of convergence due to important differences in how segments are patterned in these two clades. Recent molecular results have contradicted the idea of an Articulata clade, however, with the corollary that the segmentation seen in arthropods and annelids must either have been inherited from a much more distant ancestor, or that it is a case of convergence (Adoutte *et al.,* 2000). Likewise, recent studies seem to indicate that the apparent dissimilarities between arthropods and vertebrates might have evolved through a divergence of mechanism in *Drosophila* away from a more primitive shared mechanism.

The point we wish to establish is whether segmentation is a primitive feature of the Bilateria (i.e. of Urbilateria) and hence likely to be homologous in any group that is segmented or whether segmentation has evolved two or more times in separate metazoan clades. As highlighted above, there are two ways in which different authors have approached this problem; first using arguments from the parsimonious reconstruction of evolution of the character on the tree of the metazoans and second through attempts to demonstrate directly that segmentation in arthropods and vertebrates is homologous due to overwhelming complexity of similarities (especially molecular similarities).

The first approach is to map segmentation onto a phylogenetic tree of the Metazoa and to consider the implied costs of the two hypotheses – an unsegmented Urbilateria and multiple independent origins of segmentation or a segmented Urbilateria and multiple instances of independent loss of segmentation. This approach has been taken by Balavoine who considered a molecular phylogeny of the Metazoa and who pointed out the presence of segmentation in all three branches of the Metazoa (Balavoine 1998). Based on this observation, and supported by the shared segmental expression of the *engrailed* gene in flies and the cephalochordate amphioxus suggesting some common molecular aspects of segment ontogenesis in arthropods and chordates (see later), Balavoine concluded that segmentation was most parsimoniously reconstructed as having been present in the common ancestor of arthropods and vertebrates and that it was likely that segmentation was homologous in the two groups. Jenner has criticised this study, pointing out that the tree used by Balavoine not only contained an unresolved polytomy in each of the three main clades making it less costly to reconstruct segmentation as the ground state for the Ecdysozoa, Lophotrochozoa and Deuterostomia, but the tree also contained only a selection of metaozoan taxa and that many of those missing are unsegmented (Jenner 2000). Jenner's re-evaluation of these data on a better-resolved and more inclusive tree shows clearly that it is far from parsimonious to construe segmentation as primitive for the Bilateria or as homologous between arthropods and chordates.

Broadening the definition of segmentation

The approach of both Balavoine and Jenner leaves aside the problem of what is actually meant by segmentation; is it a single character, or can it be broken down into a set of logically separate ones, the history of each of which needs to be considered independently (Budd 2001). If considered in this way, then repeated structures (e.g. the zonites of kinorhynchs and the repeated units of many nervous systems) are much more widely spread than the typologically constrained classical examples would suggest ((Budd 2001; Balavoine and Adoutte 2003)). Both Budd and Balavoine + Adoutte consider that the fundamental features of segmentation are the repeated mesodermal somites perhaps best typified by the chordates. Balavoine and Adoutte interpret the formation of three or four paired coeloms seen in echinoderms, hemichordates, brachiopods and chaetognaths as metameric. This widening of the definition makes lack of segmentation a much rarer attribute of animal phyla and enables the parsimonious reconstruction of segmentation in the hypothetical Urbilateria.

Such considerations point to extant taxa that urgently require more careful attention paid to their molecular development. For example, if the arthropods really are the sister group of the Cycloneuralia (i.e. the clade consisting of nematodes and their relatives), then one might expect to see signs that either many "advanced" features of the arthropods, such as segmentation as well as the coelom, brain, circulatory system etc are independently gained features of the arthropods; or conversely, that the members of the cycloneuralians have *lost* these features. Sadly, the morphological development of the cycloneuralians, outside *C. elegans*, is not at all well studied, with the basic embryology of kinorhynchs, priapulids and loriciferans being entirely unknown (limited, contradictory data are available on the priapulids). As might be expected, there have been no molecular expression patterns reported from these taxa, this is despite the fact that these taxa potentially provide the basic data that would show how *Drosophila* and *C. elegans* have diverged.

Potentially, the fossil record could be of some assistance in resolving these issues, because it has the possibility of yielding stem-group forms of clades that do not yet possess all the features of the extant members. A recent example was given by Budd (Budd 2001), again on segmentation (see also Hughes 2003). Onychophorans, sister group of the euarthropods (possibly with tardigrades as even

closer relatives) apparently lack epidermal ectodermal segmentation; but this feature is known both in the euarthropods and in other segmented organisms such as annelids and chordates. Is the lack in onychophorans primitive to the whole arthropod clade, or is it a derived feature of the onychophorans themselves, perhaps deriving from their terrestrial ecology? The limited evidence available from the segmentation gene *engrailed* (Wedeen *et al.,* 1997; Eriksson and Budd, unpublished) suggests that it is not segmentally expressed in the onychophoran ectoderm; perhaps as one might expect. The large number of fossil stem-group arthropods now known reveals more of the history of segmentation in the clade. In particular, the most pertinent result is that taxa in the stem-group of the euarthropods proper also lack epidermal segmentation (Budd 1999). This interpretation which makes use of fossils and stem taxa such as the extant onychophorans and priapulids suggests that, while mesodermal segmentation may well be a primitive feature and therefore homologous between arthropods, annelids and perhaps vertebrates, epidermal segmentation of the euarthropods is by contrast a derived feature, and thus convergent on other epidermal segmentations such as that in annelids.

Of course, another possibility is that our current phylogenetic understanding is wrong, and *all* or most segmentated phyla belong in the same clade. This seems implausible; whilst there remain some die-hard proponents of Articulata clade, it seems very unlikely that the Articulata form a clade with the deuterostomes, and neither Articulata nor Articulata+Deuterostomia is supported on molecular grounds.

Genes and the homology of segmentation in arthropods and chordates

We return now to direct demonstration of homology between characters through considerations of complexity of similarity. The similarity in expression of the segment polarity gene *engrailed* in arthropods and in the cephalochordate amphioxus has already been mentioned. Balavoine and Adoutte also make mention of an arthropod-like pattern of expression, in the polychaete annelidn *Platynereis,* of its homolog of *engrailed* as well as that of another segment polarity gene, *wingless* (Balavoine and Adoutte 2003). Such a conserved expression pattern is certainly suggestive of a common and conserved role in segmentation of these genes.

It is worth noting here, however, that there is a suspicion that the cephalochordate repeated pattern of *engrailed* expression is actually a derived state within the deuterostomes as *engrailed* in vertebrates and in the hemichordates is in a single stripe within the anterior nervous system (Lowe *et al.,* 2003).

Further scepticism regarding the significance of similar patterns of gene expression comes from the observation that 87% of randomly selected genes are expressed in segmentally repeated stripes, not entirely surprising considering metamerism implies functional repetition of everything within the segment"(Liang and Biggin 1998).

More convincing support for the homology of arthropod and vertebrate segmentation comes from a recent study of spider homologs of genes in the notch signalling pathway (Stollewerk *et al.,* 2003). Arachnids make an excellent outgroup to the derived mode of segmentation in *Drosophila*. The spider *Delta-1* gene (which codes for a ligand of spider *Notch*) appears and disappears repeatedly from the posterior of the spider embryo where new segments are being formed, in a manner very similar to that previously reported for the spider *hairy* gene. Vertebrate homologs of both *Delta* and *hairy* are expressed in very much the same fashion during the patterning of zebrafish somites. Not only does the cycling of these spider genes precede segmentation, assuaging worries about meaningless segmental expression, but knockdown of these spider genes a) demonstrates an effect of the notch pathway on hairy expression as in vertebrates and b) shows disruption of segmentation and therefore likely a direct role rather simply a coincidentally segmental expression pattern(see also Peel and Akam, in press).

The molecular approach seems to hold the most promise for the resolution of this question, in particular if widened to include some of the unsegmented taxa that nevertheless have some degree of metamerism.

4. History of character evolution

Tracing character evolution

The ability to map characters onto a phylogeny means that not only can we find out the direction of evolution of character states but also, that if we are to consider more than one character, we can order their evolutionary appearance. By way of illustration, one frivolous application of this procedure has been to answer the old question of which came first, the chicken or the egg? In Fig. 7, we can see that the evolution of the characteristics of chickens is a much more recent event than the evolution of the egg which even antedates the evolution of birds, being present also in the reptiles. Clearly the egg came before the chicken.

Gene phylogenies

In general the entities in whose relationships we are interested are organism and it is generally appropriate for molecular phylogeneticists to use the phylogeny of the genes of the organisms as proxy for the phylogeny of the organisms themselves. However when genes have duplicated (and perhaps subsequently been lost), this relationship between gene phylogeny and organism phylogeny breaks down. Although this consideration means potential problems for phylogenticists, creating phylogenies of the genes themselves, and mapping this gene phylogeny onto the phylogeny of the organisms they are contained within can be informative in its own right.

One application of this tree-based approach has been mapping the changing functions of various genes underlying development. In *Drosophila*, four genes - *fushi tarazu* (*ftz*), *zerknullt* (*zen*), and the closely related *zen 2* (*z2*) and *bicoid* (*bcd*) - are clearly related to Hox genes yet lack typical Hox functions in antero-posterior patterning. Each possesses a homeobox and all four are sited within the fly Hox cluster; the prediction is that they have lost an ancestral function in AP patterning somewhere in the" *Drosophila* lineage. This conclusion has been borne out by studies of these genes in taxa at an increasing evolutionary distance from *Drosophila*. *ftz* in flies is involved in segmentation of the embryo and has a stripy expression pattern (Lawrence and Johnston 1989). In the beetle *Tribolium, ftz* is expressed in less distinct segmental stripes and, curiously, knocking it out has no effect on segmentation (Brown *et al.,* 1994). Moving further away from *Drosophila*, the *ftz* homolog in locusts has no segmental expression at all (Dawes *et al.,* 1994) and finally, when we leave the insects and look in arachnids we find the predicted primitive Hox-like expression with discrete AP boundaries (Telford 2000b). In" *Drosophila* in addition to its role in segmentation, *ftz* is involved in patterning the nervous system: a much more Hox-like role. The ability of the locust *ftz* protein to rescue the nervous system patterning function in a *Drosophila ftz* mutant but not the segmentation function suggests that, while the main AP-patterning function has been lost

this ancestral hox-like role of arthropod *ftz* has been preserved in *Drosophila* (Alonso *et al.,* 2001).

A very similar scenario is repeated with *zen*, *z2* and *bcd* all three of which turn out to be related to one another through gene duplications and to have evolved from the conserved metazoan Hox 3 genes. Again, examination of the expression of the orthologous Hox3 gene in increasingly distant arthropods shows the change in role of what was primitively a Hox gene (Telford and Thomas 1998). In the case of *zen* and *z2* the new role is in patterning the extraembryonic tissues and in the case of *bcd* there is a new role in establishing the polarity of the embryo (Akam *et al.,* 1994; Stauber *et al.,* 1999).

To continue with this theme, Hox genes are also part both of ancient and of more recent duplication events. The ancestral genes which were founder members of the anterior, central and posterior classes each have counterparts in the related parahox cluster of body patterning genes – a duplication which we are able to map to an ancestor that predates the evolution of the Diploblasts and Bilateria (Brooke *et al.,* 1998). More recently, the vertebrate Hox clusters themselves have duplicated twice (possibly as part of whole genome duplications) to give four Hox clusters in extant, jawed vertebrates (Holland *et al.,* 1994). It has been postulated that the correlation between Hox cluster (or genome) duplication and the evolution of the modern complex vertebrate bodyplan is more than coincidence.

Finally, it has long been known that the nematode *C.elegans* has fewer Hox genes than other animals such as flies and vertebrates, if, as was long believed C.elegans were an early branching animal, this lack of Hox genes *C.elegans* might represent an early stage in Hox evolution predating some gene duplications. The likely derived phylogenetic position of the nematodes, however, argues for a different conclusion; that it lacks these genes because they have been lost in the nematode lineage. Aboobaker and Blaxter use a phylogentic analysis of Hox genes within several clades of nematodes to demonstrate that this is the case. Basally branching nematodes have many of the Hox genes expected of an ecdysozoan and the authors show serial loss of Hox genes in the nematode phylogeny leading to the model *C.elegans* (Aboobaker and Blaxter 2003).

Character polarisation

In order for an analysis to be useful in an evolutionary sense, it needs also to be *rooted*, in other words we need to know the polarity of change of the characters that interest us. If we consider two taxa in isolation (say a lizard and a mouse) that differ in a certain character (e.g. hairless or hairy) how do we know which of the two has the primitive character state and which the derived? We are unable, without recourse to additional information, to determine the direction in which the evolution of this character has proceeded. The additional information needed is knowledge of the state of the character in a species that is an outgroup to the two under consideration: in this case, a frog would be appropriate. As the frog is hairless, parsimony suggests that hairlessness is the primitive character and we can infer from this that hair has evolved in the lineage leading to mice after this lineage had diverged from reptiles.

5. Explaining evolution

The approaches we have described above will allow us to reconstruct a phylogenetic tree, to determine the homology of characters in different taxa through their distribution on this tree and to map the historical timing and order of appearance through reconstruction of the ancestral nodes on this tree. The final part of our discussion examines tree-based approaches to understanding the mechanisms of evolution which will take us beyond the essentially descriptive procedures we have so far covered.

The comparative method

Much of the previous discussion has concentrated on establishing the homology of characters. The principal focus of the comparative method by contrast, is homoplastic or convergently evolved characters and the reason for studying convergence in character evolution is so that we might understand *why* a given character has evolved. The principle behind the comparative method (and here we greatly simplify part of what is a very complex field (Harvey and Pagel 1991)) is to establish a correlation between the evolution of a given character and the extraneous influence that provided the selective impetus for its evolution. In order to establish such a correlation we need more than a single observation of the co-occurrence of such and such a character with such and such an influence. To give an example of the problem and its solution, the observation that lions have sharp teeth and that they prey on other animals seem likely to be correlated but it is equally true that a lion has sharp teeth and gives birth to live young; the obvious problem is the impossibility of making a generalisation about correlation between two characteristics based on a single observation. If we went out and looked at many animals at the zoo we would probably find many more instances where there was a correlation between having sharp teeth and killing other animals but there is a high chance that our observations will have been of a cheetah, a leopard and a tiger. All of these would indeed have sharp teeth and be predators but the other correlates that depend on being a big cat would also still exist. Another way of looking at this is to note that although we may look at many different species of cat, we are only sampling a single instance of the evolution

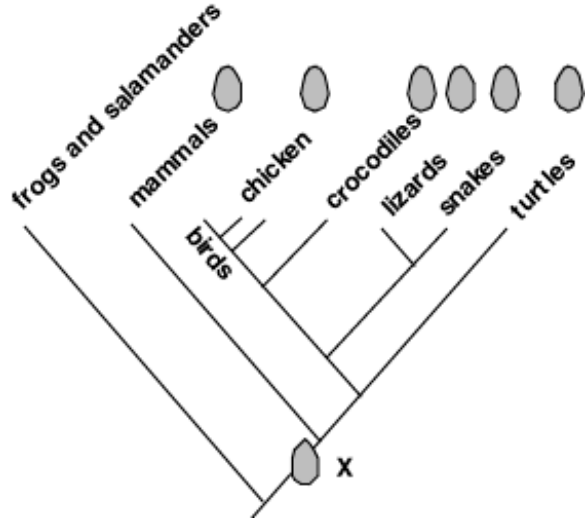


Fig. 7. Ordering the evolution of characters. *Setting the chicken in its phylogenetic context (here, a simplified tree of the tetrapods) quickly reveals that the amniote egg, of which the chicken egg is an example, is widely distributed (although it has been lost in higher mammals). The distribution implies that the amniote egg evolved at point X, well before the much later origin of the chickens. Fossil evidence suggests that the amniote egg appeared at least 310 million years ago and the first members of the Phasianidae (the family containing chickens) some 50 million years ago.*

of the sharp teeth that interest us. What we need in order to establish our hypothesised correlation is independent instances of killing animals for food, and to see if in each case there are also sharp teeth (or *vice versa*). Only when we are able to take phylogeny into account and see that we should lump cats together as one instance of the evolution of predation and then take snakes as another and the tasmanian wolf, *Tyrannosaurus rex* and sharks as further independent observations, only then can we reliably establish a correlation between the (homoplasious) evolution of sharp teeth and the (homoplasious) evolution of a predatory habit.

Linking genotypic change to phenotypic change

We now wish to consider briefly the final topic mentioned in the introduction, i.e. the general pattern of evolution as exemplified by the relationship between molecular and phenotypic evolution. We suspect that this subject will be covered in much greater detail in other papers in this volume.

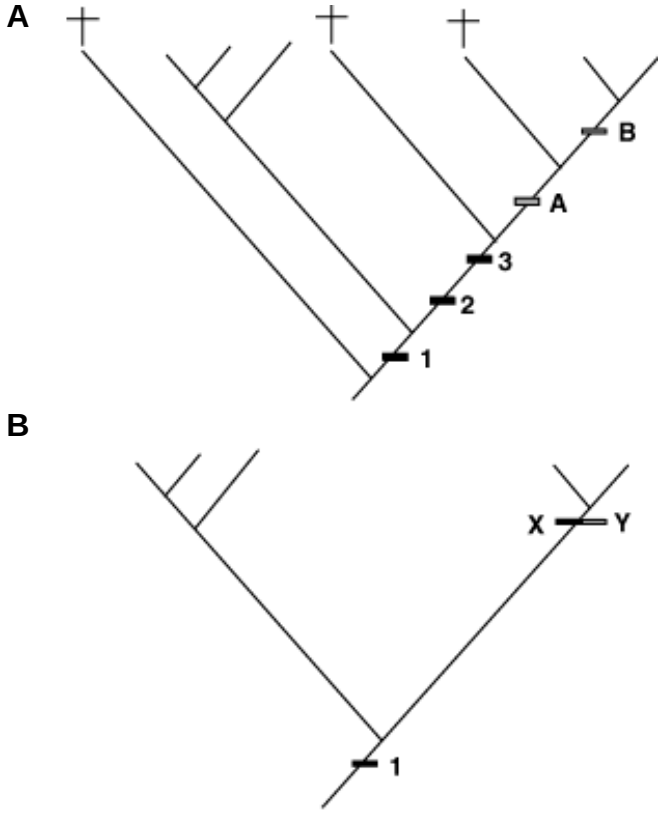


Fig. 8. Extinction and false evolutionary correlation of changes in morphology and molecules. *In* **(A)** *the true order of acquisition of molecular (grey bars, letters) and morpohlogical (black bars, numbers) changes in a clade is given. However, taxa marked with a cross are stem-group members of a sub-clade whose character states are not known. The character states may be unknown due to lack of evidence or because the taxa are extinct. Looking just at the sampled members of the entire clade shown in* **(B)** *most of the morphological (2 and 3) and all of the molecular change (A and B) is apparently compressed into a single event (X-Y) suggesting a simple cause-effect relationship between molecules and morphology. In fact, the detailed history of tree A shows the interplay between the two to be much more subtle and harder to interpret. Missing out living "minor" phyla may have a similar effect on character ordering interpretation.*

Once we are able to reconstruct ancestral states at ancient nodes in our phylogeny we can in principal progress to studies of how evolution - changes in genotype leading to the (arguably more interesting) changes in phenotype - has happened. The thinking behind the published work in this field is that if a change in morphology correlates intimately with an alteration in the expression pattern of a gene that is thought to be involved in the elaboration of that aspect of morphology then there may well be a causal relationship. If this relationship can be proved then we have progressed in our understanding of how evolution progresses by making a link between genotypic and phenotypic changes. One of the nicest examples of this approach involves correlation of changes in Hox gene expression with alterations in morphology. Averof and Patel have studied a number of crustacean taxa (Averof and Patel 1997). The primitive state of the mouthparts of these animals is to have a mandible followed by two pairs of maxillae, these three are all feeding limbs that are followed by a series of locomotary appendages or legs. The legs differ significantly in their morphology from the mouthparts and the segments bearing legs differ from those bearing maxillae in that the former express the Hox gene *Ubx*. In some crustacean groups, however, one, two or even three of the most anterior pairs of locomotory legs have altered their appearance to be more similar to the maxillae, and are now known as maxillipeds. Averof and Patel speculated that this change in morphology towards a more anterior character might have involved a change in the expression of the A-P patterning *Ubx* gene and their experiments suggest that this is indeed the case. When they look at the expression of *Ubx* in the different groups, its anterior-most border of expression always coincides with the anteriormost leg-bearing segment even when the A/P position of this alters. The anterior border of expression of *Ubx* has retracted posteriorly in those taxa where the more anterior leg appendages are now converted to maxillipeds.

In another crustacean, the woodlouse *Porcellio scaber*, a similar alteration from leg to maxilliped occurs and again Ubx is missing from the maxilliped (Abzhanov and Kaufman 1999). In this case it has also been shown that the Hox gene Scr, normally expressed in the maxillae, also appears to be involved in the transformation. In the early embryo Scr mRNA is found in both maxillae and maxillipeds but is only translated into protein in the maxillae. SCR protein is not found in the maxillipeds until later in embryogenesis and the timing of the commencement of maxilliped SCR translation correlates with the change from leg to maxilliped morphology.

Although these correlations are striking, it must be emphasised that they are just correlations. Demonstrating a direct role of the Hox genes in the transformation is relatively straightforward - misexpressing *Ubx* and removing SCR from the maxillipeds ought to transform them back into legs for example. Proving that there was a tight historical link between alteration of Hox pattern and change in morphology is less straight forward when other possibilities exist. To put it another way, the present day maintenance of a structure does not necessarily give clues as to how that structure evolved (Budd 1999). One way in which phylogenetic reconstruction can actually *mislead* investigators as to the correlation between molecular and morphological evolution is by compressing large packets of change in both molecules and morphology into the same point on a tree (Fig. 8). This comes when the process of extinction removes intermediates ("stem groups" (Budd 2001)) between extant taxa. This loss removes the possibility of reconstructing gradual sequences of change in both molecules and morphology, and therefore the true order of timing between the two cannot be determined. A classic example might be that provided

by gene duplications. For example, whilst it has been argued that genome-wide duplications may correspond to the bases of clades that show enormous diversification (e.g. the tetrapods), and are thus in some sense causal or enabling of the subsequent evolution, we are not in a position to be able to determine accurately the order of events (Holland *et al.,* 1994). Whilst it is easy to make the assumption that molecular developmental evolution is the engine that drives morphological evolution, its truth is much harder to demonstrate.

Conclusions

The critical role of phylogenetic reconstruction in drawing conclusions about the evolution of development has long been recognised. However, early attempts at interpreting evo/devo data in a phylogenetic context tended to rely on standard "text-book" views of animal phylogeny; and were not particularly concerned with the details of phylogenetic method. As the subject has matured though, much greater attention is beginning to be paid to both of these important aspects of phylogeny. As our examples have shown, phylogenetic reconstruction has been used at both large and small scales to advance various evo/devo hypotheses. Indeed, the relationship between the two subjects has grown to such an extent that evo/devo data are now regularly being used themselves for phylogenetic reconstruction. Despite these welcome trends though, phylogenetic analysis as applied to evo/devo retains some weaknesses. Arguably the most important of these is the continuing lack of coverage of "minor" taxa, which are essential for understanding the true nature of character evolution in the basal portions of clades. Many of the inferences about basal bilaterian states, for example, must be treated with caution until more developmental data is available from taxa such as the various flatworms, priapulids and lophophorates, to name a few key taxa. Phylogenetic reconstruction useful for evo/devo research may also incorporate certain types of evidence from the fossil record, above all for teasing apart critical developmental and morphological evolutionary events that would otherwise be seen as a single plexus. The striking contrast between the strict, parsimonious implications of the new trees being generated with popular hypotheses of deep homologies is a tension that lies under the surface of many phylogenetic applications in evo/devo. Finally, we would like to point to the difficulties in phylogenetic analysis itself that are particularly problematic in evo/devo research, above all those concerned with character optimization and weighting. Both these areas need to be addressed before reliable reconstruction of ancestral developmental pathways can be undertaken with confidence.

Summary

Here we review the various uses to which phylogenetic trees may be put when analysing the evolution of organisms and of the genotypic and phenotypic characteristics of these organisms. We briefly discuss the cladistic method and its application in the inference of phylogenetic trees. Next we consider the uses to which phylogenetic trees can be put: in particular for determining the homology or otherwise of characters distributed on those trees and for estimating the likely characteristics of ancestral taxa. Finally we show the application of this information for deepening our understanding of the processes of evolution. All of these forms of inference are fundamental for comparative biology and of immediate importance to the practice of evolutionary developmental biology.

KEY WORDS: *phylogeny, metazoa, urbilateria, convergence*

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