Molecular Data Indicate the Protostome Affinity of Brachiopods

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Abstract.—Although the phylogenetic position of brachiopods has always been subject to debate, many authors place them as a sister group to deuterostomes on the basis of morphological and developmental characters. However, molecular phylogeny consistently places them among protostomes. More precisely, brachiopods are predicted to branch inside the lophotrochozoan assemblage, together with annelids, molluscs, nemerteans, flatworms, and others. That result has been criticized on the basis of (1) prior knowledge of brachiopod morphology and (2) the known limitations of molecular phylogenies. Here I review recent data of molecular origin, particularly those displaying qualitative properties close to those of morphological characters. The complement of Hox genes present in all metazoa tested to date has proved to be a powerful tool for broad phylogenetic reconstruction. The mitochondrial genome also provides qualitative characters, showing discrete events of gene rearrangements. After discussing the data and the way they should be interpreted in the perspective of several hypotheses for metazoan phylogeny, I conclude that they argue strongly in favor of the protostome (and lophotrochozoan) affinity of the brachiopods. There is therefore a need for a reinterpretation of brachiopod morphological and developmental characters. I also identify some research axes on brachiopod morphology. [Brachiopod; Hox genes; mitochondrial gene order; phylogeny; rare genomic changes.]

The phylogenetic affinities of brachiopods have long been a subject of debate. In this paper, they are treated as a monophyletic group, bearing in mind that, if the brachiopods are paraphyletic and include phoronids, the conclusions drawn apply to phoronids also (Cohen et al., 1998; Cohen, 2000). Brachiopods have been classified as protostomes, deuterostomes, or an independent or intermediate third lineage of bilateral animals. Still, the classical view of animal phylogeny, based on embryological and morphological features, places them as sister group to deuterostomes (Hyman, 1940). More recent phylogenetic analyses have confirmed this placement (Eernisse et al., 1992; Nielsen, 1995; Lütter and Bartholomaeus, 1997). In contrast, molecular phylogenies have consistently placed brachiopods within the protostomes (Field et al., 1988). That result has been heavily criticized, but more careful analysis yielded the same conclusion (Lake, 1990). More sophisticated ways of analyzing sequence data have helped molecular phylogeny gain more influence, which led to a deep rearrangement of the metazoan tree shown in Figure 1 (Adoutte et al., 1999). The so-called Lophotrochozoa/Ecdysozoa hypothesis (Halanych et al., 1995; Aguinaldo et al., 1997) confirms the protostome affinity of brachiopods and places them more precisely in the lophotrochozoan assemblage, together with molluscs, annelids, flatworms, nemerteans, and a few other phyla, to the exclusion of arthropods and their affiliates. In the following, the term deuterostomes designates the monophyletic group of echinoderms, hemichordates, and chordates. Depending on the context—classical phylogeny or Lophotrochozoa/Ecdysozoa hypothesis—protostomes exclude or include brachiopods.

The placement of Brachiopods within protostomes has been criticized, not only on the basis of the morphological evidence, but also by molecular biologists, because all the trees were obtained from a single molecule, the small subunit ribosomal RNA or 18S rRNA, for which the largest sequence database is available. Moreover, because the deep branchings between metazoan phyla are close to the limit of the resolving power of this molecule (Philippe et al., 1994; Abouheif et al., 1998), it is important to test this result. Such a test is most likely to come from an emerging set of molecular characters, which originate during large genome rearrangements. These differ from primary sequence data in that they are qualitative characters, corresponding to rare or unique transformation events. Thus far, this type of character includes rearrangements of mitochondrial gene order (Boore et al., 1998), gene duplications and divergence inside broadly conserved chromosomal arrays or gene families such as the Hox cluster...
FIGURE 1. Two highly simplified phylogenies of metazoans. (a) A traditional phylogeny (simplified from Hyman, 1940). (b) The Lophotrochozoa/Ecdysozoa hypothesis (Halanych et al., 1995; Aguinaldo et al., 1997).

FIGURE 2. A summary of the Hox genes found in bilaterians. Boxes stand for genes. Horizontal lines indicate chromosomal locations, when known. Vertical white bars delineate orthologous relationships between single genes or groups of genes. Question marks indicate uncertain orthologies. The nomenclature used in this paper is indicated at the bottom.

Hox genes are a set of genes encoding developmental transcriptional regulators that make up a characteristic DNA-binding motif named the homeodomain. This 60-amino acid domain is encoded by the homeobox. Hox genes are not the only homeobox genes, but the conservation of the Hox homeodomain and a few other parts of the Hox protein allows their unmistakable identification (Gehring et al., 1994). Hox genes are found throughout the whole animal kingdom, including brachiopods (de Rosa et al., 1999) (Fig. 2). In arthropods and vertebrates, Hox genes are expressed in a sequential manner along the antero-posterior (A/P) axis of the animal during development. In phyla where they have been studied, Hox genes are usually clustered in a single chromosomal array and are ordered in a way that reflects their spatial and temporal order of expression (Lewis, 1978; Duboule and Dolle, 1989; Carroll, 1995). I have thus named them according to their expression domain and chromosomal localization as “anterior,” “central,” or “posterior” genes. The elucidation of orthology relationships between Hox genes...
of several phyla has led to the conclusion that at least seven Hox genes, and probably more, were present in the last bilaterian ancestor (de Rosa et al., 1999). After subsequent gene duplications, gene losses, or both, an average of 10 Hox genes can be found in almost every bilaterian phylum, except for vertebrates, which have multiple Hox clusters on different chromosomes, the result of several duplications of a unique ancestral cluster (Hart et al., 1987; Duboule and Dolle, 1989).

Hox genes have proved very useful in phylogenetic inference. Some Hox genes appear to have recognizable orthologs among only one phylum or set of phyla. The occurrence of such cases is often a strong argument in favor of the monophyly of the considered phyla. The first example of this use of Hox genes as phylogenetic markers was to strengthen the protostome (and lophotrochozoan) affinity of flatworms (Balavoine, 1997). To describe this argument in more details, the Hox genes are split into three groups according to their phylogenetic usefulness.

The first group comprises Hox genes that are too conserved to provide phylogenetic information. They correspond, with a few exceptions, to orthologs of the five anterior-most Hox genes (represented by paralogy groups [PG] 1 to 5 in vertebrates and lab to Scr in Drosophila melanogaster). They are easily identified in all bilaterian phyla by the presence of characteristic shared residues inside the homeodomain.

Another group comprises genes that are too divergent to have easily recognizable orthologs outside of one phylum. These are the deuterostome posterior genes (PG9–13), the insect zen and ftz, Branchiostoma flori-dae AmphiHox2, Caenorhabditis elegans egl-5, and others. In some cases, chromosomal mapping data allow identification of orthologs. They do not provide much phylogenetic information for deep branchings, but they can be useful for short-range phylogeny. For example, the insect Hox3 orthologs have undergone rapid evolution and divergence in the dipteran lineage (Falciani et al., 1996). In that case, and in other instances, the sequence divergence is correlated with the loss of the ancestral Hox expression pattern.

The last group consists of genes that are conserved enough to be recognized across several phyla but don’t have obvious orthologs in the whole bilaterian tree. They include genes of the central part of the cluster (PG6–8 in vertebrates, Antp to abd-A in D. melanogaster) and the posterior genes of protostomes (Abd-B, Post-1, and Post-2). For example, the Lox2 gene (identified by its characteristic residues: lysine at position 21, leucine at position 34, and serine at position 35 of the homeodomain; see Fig. 3) is found only in annelids, molluscs, and brachiopods. Strikingly, all the genes in that group display similar patterns of phylogenetic distribution: Some are only found in deuterostomes, some

![Alignment of central Hox genes and flanking sequences.](http://sysbio.oxfordjournals.org/)

Note the high conservation inside the homeodomain: Only a few residues allow orthology assignment. The homeodomain and the Ubd-A and Lox peptides (Balavoine, 1998) are boxed. Species abbreviations: Pca, Priapulus caudatus; Lsa, Lineus sanguineus; Lan, Lingula anatina; Pvu, Patella vulgata; Hro, Helobdella robusta; Nvi, Nereis virens; Hme, Hirudo medicinalis. AmphiHox6, 7, and 8 are Branchiostoma flori-dae genes; Antp, Ubx, and abd-A are D. melanogaster genes.
The similarity in the sequences (Fig. 4A). Alternatively, the sequences could have arisen by independent duplications in the three lineages, which would provide a synapomorphy for each of the three superphyla (Fig. 4B). Whatever the model, three character states can be defined for the Hox cluster of Bilateria.

Additional information can be found from the sequences flanking the homeodomain. The Lox2, Lox4, Ubx, and abd-A genes display a highly conserved peptide just next to the homeodomain, which is named the Ubd-A peptide. Similarly, the Lox5 genes possess a conserved peptide (Fig. 3). The Ubd-A peptide is found only in protostomes, and the Lox5 peptide is restricted to lophotrochozoans (Balavoine, 1998). The extreme conservation of each of the peptides...
makes it extremely unlikely that they arose by convergence. The existence of these kinds of peptides probably indicates that the proteins bearing them interact with a specific cofactor at this site. The gain or loss (depending on whether the peptides are ancestral or derived features among bilaterians) of one of the peptides therefore corresponds to a radical change in gene regulation at some stage of the development. It is a drastic event that must be very rare or unique.

**Mitochondrial Gene Order**

The mitochondrial DNA is a small, circular, nonnuclear chromosome. It encodes a part of the proteins used by the mitochondrion, as well as the stable RNAs used for protein synthesis. Its characteristics are quite constant in metazoans. It is circular, ~16 kb long, and comprises the same 37 genes, with very few exceptions: 2 for rRNAs, 22 for tRNAs, and 13 for proteins (Boore, 1999). Apart from a few taxa (e.g., nematodes, gastropods), the gene order is very conserved and phyla can be connected by only a few rearrangements. Although the mechanism for gene rearrangements is not known, the rearrangement events are thought to be rare. Mitochondrial gene arrangements are little prone to convergent evolution because of the large number of possible arrangements. They are therefore a very powerful phylogenetic tool to study deep branches in the metazoan tree (Boore and Brown, 1998). Several mitochondrial genomes have been partially or completely sequenced; the compiled results can be found at <http://biology.lsa.umich.edu/~jboore>.

The mitochondrial genomes sequence of two brachiopods were recently published: Teredratulina retusa (Stechmann and Schlegel, 1999) and Laqueus rubellus (Noguchi et al., 2000). Both publications conclude that the gene order of brachiopods is more closely related to that of molluscs or annelids than to chordates. Those results were based on overall similarity, without inferences of possible ancestral states. Here I will try to provide a raw cladistic analysis of the data.

The gene order of *L. rubellus* differs strongly from any gene order reported so far. It has almost no conserved gene segments homologous to those of other species, which makes the species unsuitable for the analysis. This correlates with other odd properties, such as small size, great compactness, and some peculiarities in tRNAs (Noguchi et al., 2000). Those features are obviously derived, so I will use the gene order of *T. retusa* as a representative for brachiopods, comparing the gene order of this organism with those inferred to be primitive for arthropods and chordates (Boore, 1999) and with the gene order of the mollusc *Katharina tunicata* (Boore and Brown, 1994). Because tRNA genes happen to rearrange more often than other genes, I will focus on protein and rRNA genes (listed in Table 1).

All the gene orders considered here can be connected by fewer than four rearrangements steps or six breakpoints (Blanchette et al., 1999), as shown in Figure 5. Among the gene arrangements, one is shared by molluscs and brachiopods: In both of those phyla, the (cox3-nad3) segment is linked to the (nad2-...-atp6) segment on its 3' side, but on its 5' side in chordates and arthropods. Note that the nad3-nad2 link is also found in annelids (Boore and Brown, 1995, 2000). The (cox3-nad3-nad2-...-atp6) state is thus most probably ancestral to lophotrochozoans. When comparing the three possible unrooted tree topologies for the four taxa—arthropods (A), chordates (C), *K. tunicata* (Ktu), and *T. retusa* (Tre)—the translocation of (cox3-nad3) from one end to the other of the (nad2-...-atp6) segment makes the tree ((A, C) (Ktu, Tre)) require one translocation less than the two other trees, the classical ((A, Ktu) (C, Tre)) or the unorthodox ((A, Tre) (C, Ktu)). The other translocations can be interpreted as autapomorphies and do not influence the tree topology. The tree obtained by neighbor-joining (Saitou and Nei, 1987), using breakpoint distances (Blanchette et al., 1999), has the same topology (Fig. 6).

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Abbreviation used in figures</th>
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<tbody>
<tr>
<td>Cytochrome oxidase</td>
<td>cox1, cox2, cox3</td>
</tr>
<tr>
<td>subunits I, II, III</td>
<td></td>
</tr>
<tr>
<td>Cytochrome <em>b</em> apoenzyme</td>
<td>cob</td>
</tr>
<tr>
<td>NADH dehydrogenase</td>
<td>nad1–6, 4L</td>
</tr>
<tr>
<td>subunits 1–6, 4L</td>
<td></td>
</tr>
<tr>
<td>ATP synthase subunits 6, 8</td>
<td>atp6, atp8</td>
</tr>
<tr>
<td>rRNA large subunit</td>
<td>rnl</td>
</tr>
<tr>
<td>rRNA small subunit</td>
<td>rns5</td>
</tr>
</tbody>
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Figure 5. The six pairwise comparisons for the four mitochondrial gene arrangements of Arthropods, Chordates, Katharina tunicata, and Terebratulina retusa. The gene order maps are arbitrarily started from cox1. A minus before the name indicates a gene on the opposite DNA strand. One of the minimal sets of rearrangements is proposed for each comparison. The number of rearrangements needed (and the breakpoint distances [Blanchette et al., 1999] inside parentheses) are indicated on the left.

Figure 6. Unrooted neighbor-joining tree obtained by using breakpoint distances.

DISCUSSION

Analysis of Qualitative Molecular Evidence

The Hox and mitochondrial data can be fit only into a single unrooted tree (shown in Fig. 7A). Any other tree requires additional convergent steps, which, given the rarity of the events considered here, is highly unlikely. Of the five possible rooted trees, three (Fig. 7B–D) propose a brachiopod/mollusc sister grouping, which confirms the existence of lophotrochozoans. The tree in Figure 7B, which corresponds to the Lophotrochozoa/Ecdysozoa hypothesis, is the only one that retains protostome monophyly. A paraphyletic lophotrochozoan assemblage remains possible, if one assumes that the lophotrochozoan Hox cluster retained the ancestral state (Fig. 7E, F). In that case, however, one has to assume a chordate/arthropod
(or ecdysozoan/deuterostome) sister grouping (also found in Fig. 7D), which, to my knowledge, has never been proposed.

Although not definitely conclusive, the data strongly suggest that lophotrochozoans are a natural group and exclude a close relationship for brachiopods/deuterostomes. A definitive confirmation would require a rooted tree, which so far has been impossible to obtain, because the Hox family and mitochondrial gene order in cnidarians, the likely sister group to bilaterians, differ greatly from their bilaterian counterparts and are thus far impossible to connect to one or the other of the character states described here. Perhaps this issue could be solved with data from the ctenophores, but so far such data are not available. In the meantime, considering the incoming wealth of independent sequence data from 18S rRNA (Halanych et al., 1995; Aguinaldo et al., 1997) and mitochondrial gene sequences (Stechmann and Schlegel, 1999), together with the qualitative data analyzed here, I consider the Lophotrochozoa/
Ecdysozoa hypothesis to be the most probable depiction of bilaterian history.

The Phylogenetic Significance of Brachiopod Morphological Features

On the basis of this phylogeny, the need for a reappraisal of most of the morphological and embryological characters of brachiopods is obvious. It is not in the scope of this short review to make a complete reinterpretation of the data accumulated over more than a century. I would like only to point out a few characters that, in light of the lophotrochozoan affinities of brachiopods, must have been misinterpreted. General morphological and developmental considerations in this part are mostly taken from classical zoology and development textbooks (Anderson, 1973; Brusca and Brusca, 1990; Meglitsch and Schram, 1991; Nielsen, 1995; Gilbert, 1997; Wolpert, 1998).

Egg cleavage patterns.—The question of cleavage patterns has been discussed elsewhere (Valentine, 1997) and can be summarized as follows: Radial cleavage is most likely ancestral for Bilateria and therefore cannot be used to unite brachiopods with deuterostomes. The conservation and complexity of the spiral cleavage pattern make it very unlikely to have arisen several times independently. This pattern is restricted to annelids (sensu lato, including echiurans and pogonophorans [McHugh, 1997]), entoprocts, molluscs, nemertines, platyhelminthes, sipunculids, and a few small phyla, which could be a sister group to the brachiopod/phoronid clade among lophotrochozoans. Alternatively, if brachiopods branch inside the spiralian protostomes (Cavalier-Smith, 1998; Giribet et al., 2000), or if spiral cleavage is ancestral to protostomes (if the occurrence of a modified spiral cleavage in arthropods [in crustaceans or pycnogonids or both] is a correct interpretation), then spiral cleavage must have been lost in brachiopods and other lineages. Many shifts from spiral to radial to idiosyncratic cleavages are known (e.g., acoel flatworms, cephalopods, nematodes, onychophorans, pogonophorans, and many arthropod and vertebrate groups), making it appear that the cleavage pattern is a highly variable character. That pattern is connected to the mode of fertilization, the yolkiness of the egg, and the environment in which the embryo develops. Given its high contribution to species fitness and its great variability, cleavage pattern is not a reliable character for deep metazoan phylogeny.

Modes of coelom formation.—The classical split (schizocoelic protostomes vs. enterocoelic deuterostomes and brachiopods) is also subject to criticism. Brachiopods display both modes of coelom formation, showing that this character again is prone to important variation. Budd and Jensen (2000) argue that the different modes of coelom formation can be explained by differences in the timing of development. The plasticity of the development of body cavities has long been recognized (Ruppert, 1991), and some have proposed that the mode of coelom formation is highly correlated with cleavage pattern (Valentine, 1997). What was said above about cleavage pattern can thus be extended to coelom formation: It does not appear to be a character suitable for deep metazoan classification.

Protostomy versus deuterostomy.—The fate of the blastopore and the origin of the mouth have also been used as characters unifying brachiopods and deuterostomes. This character is also highly variable and should not be used for uniting phyla (Nielsen, 1995). Because amphistomy (blastopore giving rise to mouth and anus by lateral closure) is probably the ancestral state for Bilateria (Arendt and Nubler-Jung, 1997), deuterostomy (formation of a secondary mouth independent of the blastopore) would be a derived character. However, deuterostomy unquestionably is found in protostomes, such as the polychaete Eunice and the gastropod Viviparus (Anderson, 1973; Arendt and Nubler-Jung, 1997), and therefore is prone to convergence and cannot be used to link brachiopods and deuterostomes sensu stricto.

The three examples above illustrate the danger of reasoning on archetypes (radial-cleaving enterocoelous deuterostomes vs. spiral-cleaving schizocoelous protostomes) rather than seeing the diversity of characters among phyla. This practice can lead to overestimation of the conservation of very labile characters. The three characters mentioned above are unsuitable to solve the phylogeny of bilaterians. They might still be useful for phylogenies at a lower taxonomic level.

Trimery.—Among the other characters proposed to unite brachiopods and deuterostomes are a trimeric coelom and a tentacle
feeding system connected to the second pair of coelomic sacs. Concerning the feeding apparatus, characteristics that would definitely prove the homology of the brachiopod lophophore with the tentacle feeding system of some hemichordates (pterobranchs) and echinoderms are not known (Halanych, 1996). Additionally, because hemichordates and echinoderms are most probably sister groups (Wada and Satoh, 1994; Halanych, 1995), there is no evidence that a sessile adult with tentacular feeding apparatus is the ancestral state for deuterostomes. More probably, a sessile adult stage appeared independently in pterobranchs (Cameron et al., 2000) and urochordates (Swalla et al., 2000). Some annelids display similar feeding systems acquired independently. Again, this character seems prone to convergence. The number of coelomic sacs is also a highly variable feature. Cephalochordates have many pairs of coelomic sacs and some brachiopods have only two. The number of occurrences of a repeated structure is not a very strong character: The ability to replicate a structure makes modifying the number easy. Annelids and arthropods, for example, do not always display the same number of segments. The number of different categories of vertebrates is variable among vertebrates. A small number of coelomic sacs could easily be reached convergently, by either the splitting of an original pair of coelomic sacs or the loss of segments from a segmented ancestor. The origin of brachiopods from an annelid-like segmented ancestor has already been proposed, on the basis of a completely different argument (Gutmann et al., 1978). Again, the phylogeny of Bilateria is not a taxonomic scale at which this character can be useful.

Larval feeding apparatus.—Larval ciliary bands used for filter-feeding have been used to unite brachiopods and deuterostomes (Nielsen, 1987). Protostomes have a downstream collecting system formed of compound cilia, whereas deuterostomes and brachiopods have an upstream collecting system formed of single cilia. Although no filter-feeding outgroup exists to prove this, and although the function of the character is not well understood, the complexity of an upstream collecting system causes authors to consider it a unique derived feature (Nielsen, 1995). In that case, downstream collecting systems are considered plesiomorphic (but see Rouse [2000] for a different point of view). An upstream collecting system would therefore be a synapomorphy of deuterostomes and brachiopods. I propose a different interpretation. Because downstream collecting systems are found only in protostomes, it is tempting to consider them as blastopore ciliary systems rather than mouth ciliary systems. Additionally, the same kind of compound cilia are found in the perpendicular ring of amphistomous trochophore larvae. In animals that form a secondary mouth, a different ciliary system must have been recruited. Suppose the competence to form a downstream-collecting system with compound cilia were restricted to the edges of the blastopore. The upstream collecting system would then be convergently recruited (for example, from locomotory ciliae, to which they are very similar) in animals forming a secondary mouth. It would thus be very interesting to study the ciliary bands in unquestioned protostomes that form a secondary mouth. Unfortunately, to my knowledge, neither Viviparus nor Eunice has filter-feeding larvae for testing this prediction, so this interpretation remains speculative.

Larval nervous system.—Hay-Schmidt (2000) has classified the serotonergic nervous system of larvae into six types. Type 1 is restricted to cnidarians, types 2 to 4 are found in spiralians, type 5 is found in lophophorates and nonchordate deuterostomes, and type 6 is in chordates. Let me stress that this pattern does not support a brachiopod/deuterostome relationship (Fig. 8). Similarly, any character having the following distribution pattern—state A in spiralians, state B in deuterostomes and lophophorates, and other or unknown states in diploblasts and ecdysozoans—is not an argument favoring a brachiopod/deuterostome grouping, because B could be plesiomorphic for bilaterians.

Larval development.—Brachiopods have many different idiosyncratic larval types.
Some early brachiopod larvae have mantle lobes (Terebratulina transversa; Freeman, 1993), a shell (Glottidia pyramidata; Freeman, 1995), or a lophophore (Discinisca strigata; Freeman, 1999). They hardly undergo metamorphosis, and I prefer to name them direct developers. Because indirect development is most likely ancestral in bilaterians (Nielsen, 1995; Peterson et al., 2000; Arendt et al., 2001), a phylum that includes both modes of development suggests direct development is derived inside the phylum. The planktotrophic larva of Crania anomala does not display obvious adult features and undergoes complete metamorphosis (Nielsen, 1991). Therefore I consider C. anomala to be closer than other species to the ancestral state for brachiopods. For instance, the full-grown larva of C. anomala, with three pairs of setal sacs related to coelomic cavities, displays strong similarities with some larvae of polychaete annelids. The setae of brachiopods are identical to those of annelids (Lüter and Bartholomaeus, 1997). Although overall similarity is not a sound basis for classification, this agreement remains suggestive. Additionally, Nielsen (1991) has proposed that the larva of C. anomala transiently forms four pairs of coelomic sacs. If this interpretation is correct, it makes more sense to consider that brachiopods have had a segmented ancestor than to imagine that this transient structure is a derived state for C. anomala.

Conclusion

A whole set of independent molecular characters, including qualitative data much less prone to known artifacts than primary sequence data, point toward making the Lophotrochozoa/Ecdysozoa hypothesis the best depiction of bilaterian phylogeny. This implies that brachiopods are protostomes. As I have argued here, the characters supposed to unite brachiopods and deuterostomes must have been misinterpreted: Most of them can be shown to be either plesiomorphic to Bilateria or prone to convergence. A few morphological and developmental characters do not explicitly support the inclusion of brachiopods inside protostomes, but no character overtly conflicts with this proposal either. Brachiopods should therefore be considered protostomes.

ACKNOWLEDGMENTS

Some of the ideas expressed here came to me during lectures or discussions at the 2nd TMR course in Evolutionary Developmental Biology held in Roscoff in May–June 2000. I thank the organizers, the participants, and the funding agencies of the course. I thank André Adoutte, Guillaume Balavoine, Olivier Lepinet, Richard Olmstead, and two anonymous reviewers for helpful discussion and comments on the manuscript. The work in Gif-sur-Yvette is supported by the Centre National de la Recherche Scientifique and the Université Paris-Sud.

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Received 7 November 2000; accepted 5 February 2001

Associate Editor: R. Olmstead