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Evo-Devo: the Long and Winding Road

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ABSTRACT Evolutionary developmental biology (Evo-Devo) aims to unveil how developmental processes and mechanisms become modified during evolution and how from these changes the past and present biodiversity arose. The first wave of Evo-Devo identified a conserved set of toolkits common to most metazoans. The present second wave has changed gear and aims to identify how genes and modules were used differently through evolution to build the past and present morphological diversity. The burgeoning third wave is introducing experimental testing of predictions drawn from the first and second waves. Here we review some of the hottest topics, contributions and insights of present Evo-Devo related to basic concepts and paradigms of evolutionary research. Future directions of Evo-Devo are also highlighted; in other words, *Quo Vadis,* Evo-Devo?

KEY WORDS: *biodiversity, homology, gene network, macro-evolution, experimental Evo-Devo*

"To invent from nothing an animal that can exist (I mean to say that can physiologically grow, nourish itself, resist the environment and predators, and reproduce itself) is an almost impossible feat. It is a project that by far exceeds our rational abilities and also that of our best computers: we still know too little about existing vital mechanisms to create others, even only on paper. In other words, evolution has proven itself to be enormously more intelligent than the best evolutionists. Every year that passes confirms the fact that the mechanisms of life are not exceptions to the laws of chemistry and physics, but at the same time the furrow which separates us from the ultimate comprehension of vital phenomena grows ever wider. It is not that problems are not solved and questions not answered, but every solved problem generates dozens of new ones and the process gives no sign of ending."

Primo Levi

'Inventing an Animal' (1985) Other People's Trades (tr. Raymond Rosenthal, 1989)

"The great destroyer: Time"

E. Ray Lankester

Annals and Magazine of Natural History (1870)

"The Cambrian "explosion" of metazoans and molecular biology: would Darwin be satisfied? Fascinated, certainly; but satisfied? Not yet!"

Simon Conway-Morris

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In his book "Embryology and Genetics", Thomas Hunt Morgan (1934) advanced what is considered to be the first rational explanation of how cells differentiate along embryonic development. His argument was as follows: because egg cytoplasm is heterogeneous, the equipotential nuclei will be found, after several cleavage divisions, in different cytoplasmic environments which will turn on different sets of genes. This will, in turn, change the cytoplasm of each cell increasing differences among them. Although he did not use such phrasing, this was the first clear presentation of the basic tenet of Developmental Genetics to explain embryonic development: differential gene expression in time and space. At that time, one might have had the impression that understanding embryonic development was at hand. Actually it took more than 50 years to start understanding it and today, large gaps still remain unexplored.

Because morphological evolution could be simply stated as the evolution of regulation of the genetic 'toolkit', together with some pinches of gene duplication and gene diversification, one might also be tempted nowadays to conclude that we are close to understanding morphological diversity and evolution. Indeed, in the last 30 years, developmental genetics and molecular biology have uncovered the main building blocks to explain evolutionary changes in development. Developmental regulatory genes and basic cellular processes (differentiation, apoptosis, morphogenesis) and cellular properties (proliferation, migration, cell-cell interactions) have been identified and shown to be used in all organisms during evolution to provide the genetic and cellular bases for evolutionary change. Moreover, the tremendous stride of molecular biology and genetic engineering techniques is providing genetic lists from increasing numbers of species. Measurements on a global scale of changes in concentrations of thousands of mRNAs and proteins, and the development of new analytical techniques to

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identify potential interactions among cellular and genetic components have also ensued. However, even to the most unbounded optimist, we are still far from understanding morphological diversity and evolution. The excellent contributions which make up this *"Evolution & Development"* Special Issue of the *Int. J. Dev. Biol.* are evident witnesses of progress; even so, the concern remains that most explanations fall short of providing final answers to most of the questions posed! In what follows, we list what we consider to be the main problems facing Evo-Devo and some of the potential ways out.

1) New building blocks: did sponges and cnidarians invent everything? Is there "life" beyond gene duplication and diversification?

Despite being morphologically very diverse, multicellular organisms are made by a very conserved set of regulatory genes playing comparable developmental roles. This most unexpected finding represented a powerful molecular proof of evolution as 'descent with modification' (Darwin, 1859). Nonetheless, it furnished a big paradox: if developmental genes are the same, how are differences in development and in the final morphology in different organisms to be accounted for? After the shock, the likely answer: differences between close organisms are due to differences in expression of regulator genes driven by upstream regulators or by changes in the range of downstream target genes.

So far, so good. But what does this mean and how should the issue be tackled? Whereas it is true that sponges already bear scores of protein kinases, G proteins, phosphatases and so on (Suga *et al.,* 1999, 2001), it is also true that they lack key elements of the metazoan toolkit such as *Hox* and *ParaHox* cluster genes and the main 'mesodermal' genes. A bit further up, and despite featuring a simple structure with a single body axis and radial symmetry, Cnidarians bear the main elements of the genetic toolkit (Finnerty and Martindale, 1999; Hobmayer *et al.,* 2000; Spring *et al.,* 2002; Scholz and Technau, 2003; Yanze *et al.,* 2001; Hayward *et al.,* 2002; Kozmik *et al.,* 2003; Seipp *et al.,* 2001, Wikramanayake *et al.,* 2003). In other words, *Hox/ParaHox* gene clusters, several anteroposterior (or AP) genes (*Otx, emx,..*), the main set of endodermal and mesodermal genes (*Brachyury, Fork Head, snail/slug, twist, MyoD, Mef2, dpp/BMPs, Wnt/ß-catenin,…*), *Pax* genes, germ cell genes (*vasa, nanos*), as well as several genes involved in apoptotic processes, were already in place 600 million of years ago. Evidence of such an extensive toolkit in sponges and, in particular, in Cnidarians debunks the notion that gene diversification was at the base of the so-called Cambrian explosion, whatever this may mean today (see Conway-Morris, 2003).

If from cnidarians onwards the basic toolkit remained within the same order of magnitude, several processes may have underlaid some of the big changes brought about from the Proterozoic: 1) temporal changes in the amplitude of expression and spatial changes in the location of the regulatory states brought about by changes in cis-regulatory elements or by incorporation of new cis-regulatory regions in key genes resulting in new and specific changes in patterning or in cell fate and function; 2) cellular processes (cell migration, programmed cell death,..) considered as cassetes and co-opted over and over to provide the "cellular" basis for evolutionary change (Rudel and Sommer, 2003); and 3) gene duplication and diversification resulting in similar but somehow different roles (e.g. gene families during chordate evolution).

A final concern: is it true that there is no life in Evo-Devo besides gene duplication and diversification? In other words, did animals invent nothing after they became multicellular? Was everything a question of shuffling, tinkering and co-option? Data from the increasing number of fully sequenced genomes indicates a substantial number of novel unmatched genes (Pires-DaSilva and Sommer, 2003). What are they for?

2) The need for accurate phylogenies for meaningful Evo-Devo questions

Evolutionary-based questions have to be framed into a meaningful phylogenetic framework; otherwise, whether a particular morphology is ancestral or derived, whether a new morphology is due to gain or loss of a feature, or whether a morphology has evolved once or many times can not be properly answered unless phyletic relationships among the comparing clades is known. Jenner (2000) has cogently pointed out how incomplete taxon sampling and/or prunned phylogenies invalidate the most basic features (coelom, segments and indirect life-cycle as ancestral characters) called upon to back two recent hypotheses on the origin and evolution of bilaterian metazoans: the so-called "complex Urbilateria" hypothesis (De Robertis, 1997; Holland, 2000; Holland *et al.,* 1997; Adoutte *et al.,* 1999, 2000) and the "set-aside cells" hypothesis (Peterson *et al.,* 1997; 2000).

Another interesting case is the presumed homology, based on the 'overwhelming' complexity of similarities (especially molecular similarities), between annelid, arthropod and vertebrate segmentation (Holland *et al.,* 1997; Balavoine, 1998; Adoutte *et al.,* 1999, 2000). Amidst other advanced features, the ancestral bilaterian was posited to be segmented. This implied the unparsimonious loss of segmentation in more than a dozen unsegmented phyla. Since neither morphological nor molecular phylogenies have so far been able to sort out the cladogenetic relationships of these unsegmented phyla among themselves and with the three segmented phyla within the Lophotrochozoa, Ecdysozoa and Deuterostomia, whether segmentation is an ancestral or a derived character remains an open question. Central to this uncertainty is what segmentation actually means (Budd, 2001a) and whether its meaning has to be widened to accommodate any repetitive feature (e.g. see Balavoine and Adoutte, 2003), a proposal a bit farfetched. Instead of claiming homology on the basis of "similar" patterns of gene expression between clades that diverged at least 500 million of years ago, it is more advisable to undertake two complementary approaches: 1) use EST collections and/or multigenic approaches, to get the 'final' phylogeny of the Bilateria and 2) undertake deeper cellular and molecular analyses to track adult vestiges or embryonic rudiments of segmentation in unsegmented clades (Telford and Budd, 2003). Such approaches must also be used to tackle the hottest points in animal evolution (see 8: a hot *Evo-Devo* Agenda).

Within individual phyla, classes and orders, phylogenies have provided safer and better solutions. The identification of the sister-relationships of the insects and crustacens (Friedrich and Tautz, 1995) has helped to interpret developmental and morphological differences among them. In parallel, analyses of mitochondrial genomes have been instrumental to cluster the Phoronida within the Brachiopoda, the Acanthocephala within the Rotifera, and to show the proximity of Echiurida and Pogonophora to the Annelida. Finally, phylogenies are becoming extremely important for analyz-

ing whether a morphology has evolved or de-evolved once or several times and whether complex traits can be re-evolved by lineages that lost them. Three nice examples are: the recent claim, based on character distribution mapped onto molecular phylogenies (Whiting *et al.,* 2003), concerning the repeated re-evolution of wings in stick insects (but see an interesting rebuttal by Stone and French, 2003); the loss and regaining of limbs during the evolution of the major clades of snakes (Greene and Cundall, 2000), and the parallel evolution of direct development in sea-urchins from ancestral indirect developers (see Sly *et al.,* 2003).

3) How to sort out homologies from convergent evolution, parallel evolution and homoplasies

The debate about what homology is, continues. Brian Hall's book devoted to homology (Hall, 1994) lists 19 types of homology. The father of the homology concept, Richard Owen, gave in 1843 his last formal definition of homology: *"Homologue….the same organ in different animals under every variation of form and function"* (Owen, 1843). Owen contrasted homology with analogy *"a part or organ in one animal which has the same function as another part or organ in a different animal"* (Owen, 1843). The traditional view of morphologists is that morphological similarities may be due to either homology or homoplasy, a term introduced by Lankester (1870) to incorporate an evolutionary dimension into Owen's definition. Nowadays, one definition that would be accepted by most, although not by all, could be the occurrence of the same feature in two organisms, whose common ancestor also possessed the feature. Hence, homologous structures arise by common descent from an ancestral form, whereas homoplasious structures between two organisms are independently derived in the respective lineage, either by parallel evolution (similar morphological solutions using similar ontogenetic mechanisms) or by convergence (similar morphological solutions using distinct ontogenetic mechanisms).

The most exciting and far-reaching discovery of modern Evo-Devo was the finding that many developmental genes are conserved among deeply divergent taxa. So, organisms as different as nematodes, flies and mammals use similar genes for similar developmental purposes. Ever since, the study of homologous genes has been widely used as a clue to identify homologous developmental processes and structures. Indeed, several examples of using such approach are included in this Special Issue to investigate major macroevolutionary events, e.g. the origin of segmentation (Seaver, 2003), germ layers (Technau and Scholz, 2003), the central nervous system (Ghysen, 2003), or the origin of eyes (Arendt, 2003). However, as early embryologists and geneticists already foreshadowed, another fascinating realization from the past two decades is the extraordinary complexity of the relationship between genotype and phenotype. The link between the genetic makeup of an organism (its genotype) and its form and function (its phenotype) lies at the heart of evolutionary developmental biology. Such a gap, or black box, calls for extreme caution when developmental homologies are inferred from gene homologies. It is now clear that distinct kinds of dissociations can evolve between homologous genes and homologous aspects of morphology (Wray and Abouhief, 1998, Rudel and Sommer, 2003, Hall 2003).

Homologous genes may be responsible for non-homologous morphologies. The more we known about regulatory genes, the clearer it becomes that few genes are devoted to a single developmental task. Development proceeds by the coordinate implementation over time of a scaffolding of gene cassettes and networks, which are utilized on many separate occasions during development. As examples, the *Notch*-signalling pathway is broadly used during *Drosophila* and vertebrate development (Simpson, 1997; Robey, 1997), and *hedgehog, TGF-β* and *Wnt* family members are used over and over again during development. Evolution may well have worked by "genetic tinkering" (Jacob, 1977) or "bricolage" of gene networks (Duboule and Wilkins, 1998). This tinkering or "bricolage" may result in the recruiting of the same developmental module in evolutionary disparate structures. Gene expression or action will then not reflect homology but recruitment. No one will posit *Notch* expression to argue for homology between the vulva in *C.elegans,* the *Drosophila* eye and the presomitic mesoderm of vertebrates (Wray and Abouiheif 1998, Pourquie, 2003), or argue for homology between the neural tube of vertebrates with the wing of arthropods based on *hedgehog* function. However, the expression of *engrailed* in stripes in the first eight somites of the cephalochordate amphioxus (Holland *et al.,* 1997) has been used as an argument to support the monophyly of segmentation in bilaterians (reviewed in Seaver, 2003), whereas the expression of the *Hox, Otx* and *Emx* transcription factors has been advanced to homologize the central nervous system of arthropods and chordates (Sprecher and Reichert, 2003; Ghysen, 2003).

Gavin de Beer already noted that homologous genes and homologous structures could be dissociated ("characters controlled by identical genes are not necessarily homologous", de Beer, 1971). In modern Evo-Devo, the rule is that the deeper the phylogenetic distance, the more complicated it is to distinguish whether a similar structure dictated by a similar gene network is homologous or is the result of parallelism, or independent recruiting of a gene network for similar purposes. Sorting out parallelism from homology in landmark issues such as the origin(s) of segmentation, the coelom or the origin(s) of the eye (Hodin, 2000) warrants further studies of additional phyla, and a deep understanding of plasticity and constraints in gene and developmental evolution. De Beer also noted the inverse situation: *"homologous structures need not to be controlled by homologous genes"*. Although few examples of this phenomenon have been described, e.g. the segmentation role of *Evx* in Schistocerca (Patel *et al.,* 1992) and the posterior vulva formation in nematodes (reviewed in Rudel and Sommer, 2003), when a structure which is homologous between closely related organisms is built using different genes, it should be considered convergent rather than homologous.

Nonetheless, the use of expression and function of homologous genes to infer morphological homology between relatively close related species has been widely and successfully used, and in many cases, has given remarkable insights into the appearance of developmental innovations (e.g, Wada *et al.,* 1999, Ferrier *et al.,* 2001). However, our understanding of the role of parallel evolution to produce similar structures and the mechanisms for the occurrence of convergent evolution is far from satisfactory. A future challenge to approach the intimate rules (if any) governing convergence and parallelism in animal evolution will require detailed case-by-case studies in closely related, "satellite species" of well documented animal models (Rudel and Sommer 2003). Yet, comparing genes and gene networks between species, particularly species at relevant crossroads of animal evolution, will be in

the forthcoming years a major generator of information for grasping principles at the base of homology, convergence and the evolution of developmental mechanisms (Davidson *et al.,* 2003; Hinman *et al.,* 2003; Revilla-i-Domingo and Davidson, 2003).

4) Is macroevolution something special or just cumulative microevolutionary changes?

The "first wave" of Evo-Devo uncovered the great deal of similarities in genes and cellular processes shared by all organisms (Gilbert, 2003). The present "second wave" has changed gear with the aim of identifying differences in how these gene and modules are used. Several examples documented in recent years are worth mentioning: i) mutations in protein coding sequences. A mutation in the Ubx protein represses *Distal-less* expression in insects but not in other arthropods, resulting in insects bearing only thoracic legs (Galant and Carroll, 2002; Ronshaugen *et al.,* 2002); ii) alterations in genes down-stream of regulatory genes. Whereas Ubx represses growth in the dipterans halteres, it does not in butterfly hindlimbs, resulting in two wings in the former and four in the latter (Weatherbee *et al.,* 1998); iii) altering the spatial or temporal expression of regulatory genes. The spatial shifts of regulatory genes seem to be involved in morphological changes, such as the type of appendages formed in crustaceans, the webbed feet of duck, the axial formula derived from somites, or the loss of limbs in snakes (Cohn, 2002, Gilbert, 2003, Rudel and Sommer, 2003). Heterochronic switches of expression patterns may account for evolutionary changes in vertebrate limbs and polyphenism in insects (Smith, 2003).

Nonetheless, the major evolutionary transitions in animal evolution still remain to be causally explained. The origin(s) of multicellularity, the origin of bilateral symmetry, the radiation at the Cambrian Explosion and the origin of vertebrates are the most relevant macroevolutionary events for which no genetic thunderstorm may be invoked, the only exception being the concurrent poliploidization events at the onset of vertebrate origins and early steps of vertebrate evolution (Holland *et al.,* 1994). Macroevolutionary processes are extensively analyzed elsewhere in this volume (Ferrier and Minguillón, 2003; Conway-Morris, 2003; Valentine and Jablonski, 2003; Finnerty, 2003; Technau and Scholz, 2003) and early steps of metazoan evolution are considered below.

As Dobzhansky (1937) firstly pointed out, the main issue in the macro *vs.* microevolution debate is whether mutations resulting in real evolutionary novelties are of the same kind as those occurring daily or whether we should expect special, rare mutations only occurring on geological time scales. In the late 60s, population and quantitative genetics showed a high deal of genetic variation within populations. Evolutionary developmental biologists thought of this intraspecific variation in regulatory developmental genes as mere "noise". However, new applications of population genetics and artificial selection techniques to test the potential of variation in developmental features is swiftly changing this appreciation. Six generations of artificial selection on wing eyespot size in the butterfly *Bicyclus*, led to dramatic shifts in the range of eyespot sizes (reviewed in Beldade and Brakefield, 2002). Further, Gompel and Carroll (2003) and Sucena *et al.,* (2003) have identified minor genetic changes correlating with microevolutionary features in closely related *Drosophila* species: the distribution of tricomes, or the pigmentation of the abdomen.

Therefore, population genetics, quantitative genetics and natural variation are approaching developmental genetics and Evo-Devo. A tentative early conclusion is that variation is there, not necessarily hidden, but underused or plainly not used. Evolution, even at higher levels, may proceed by adaptative selection of variation of developmental regulatory genes. This variation may endow bricolage of modules or toolkits, bricolage that may result in successful morphological innovations. Theoretical biology and computational (digital) organism studies show that complex systems may be generated by synergy of a limited number of genes or simple modules (Solé *et al.,* 2003). Meanwhile, populations of simple digital organisms (computer programs that self-replicate, mutate, compete and evolve) often evolve the ability to perform complex logical functions (Lenski *et al.,* 2003). Translating these theoretical studies to real life and evolution may be difficult to handle. However, they charmingly imply that no entirely new genetic component will be found to be responsible for morphological innovation; rather, subtle changes and combinations within or between existing toolkits will be the actual cause.

However succesful such microevolutionary explanations turn out to be, non-random origination in time of evolutionary novelties seems out of reach of 'simple' explanations based on polymorphic changes in regulatory regions or shifts in gene frequencies. In addition, successful innovations require contingent events such as ecological opportunity, developmental possibilities and appropriate environmental settings. As it stands, microevolution does not provide a satisfactory explanation for the extraordinary burst of novelty during the Cambrian Explosion (Erwin, 2000). Repatterning and redeployment of pre-existing developmental potential within a novel ecological milieu may have been largely responsible for the radiation (Erwin, 2000; Conway-Morris, 2003), but invention of new genes, extensive or non-extensive gene duplication and the expansion of particular gene families, such as *Hox* genes, have yet to be fully explored (Ferrier and Minguillón, 2003).

Another stumbling block to get a balanced assessment of macroevolution is the excesive, almost mystical, adherence to typological concepts such as Baüplan and phylum which are preformationist and pre-evolutionary. Such concepts muddle and distort the perception of big radiations (the paradigm is the so-called Cambrian Explosion, though it could be extended to the radiation of land plants, mammals, etc,…) leading us to see them as something amazing, exceptional and unique, which they were not, and needing exceptional mechanisms, which likely were not required. Budd (2001b) and Fitch and Sudhaus (2002) have cogently argued (see also Conway-Morris, 2003) that such perceptions are the result of bad systematics ('stem groups' or fossils are usually left out) and of not considering that with elapsed time both the disparity among clades and the opportunity for extinctions of intermediate forms increase. Skipping the fossil record removes the 'stem groups' (those between the most recent common ancestor of two living groups and that of only one of them), which must comprise, by definition, only fossil organisms. This leaves for comparison only 'crown groups' (the most recent common ancestor of a clade plus all of its descendants) which are of little help, especially when comparing high clades (e.g. phyla). This is because lineages diverged from each other in a step-by-step manner which is only documented in the fossil record. In addition, ignoring that elapsed time increases the opportunity for intermediate forms to be extinct, reinforces the mirage that extant

'crown groups' (usually phyla) appeared at once in their present modern form.

The future task for macro-Evo-Devo will be to unravel: i) pre-existing developmental potential; in other words, what was before the Cambrian, which means analyzing the developmental toolkit component of the closest sister groups, relatives of eubilaterians, the acoelomorph flatworms (Ruiz-Trillo *et al.,* 1999; 2002); ii) the extent and quality of bricolage of this basal bilaterian toolkit compared to higher bilaterians; and iii) trying to link the known, and those which remain to be discovered, fossil groups (that is, the 'stem groups'; Budd, 2001b) to the extant 'crown groups'. Other major macroevolutionary events, such as the origin of multicelularity and the origin of bilaterians need similar grounds; a profound analysis of the relevant sister relatives and the inclusion of relevant fossil data. Data concerning Cnidarians is growing indeed (Finnerty and Martindale, 1999; Spring *et al.,* 2002), but sponges, ctenophores, placozoan, mesozoans and other minor and often unclassified phyla need to jump into Evo-Devo to help us to understand better the how(s) and why(s) of macroevolution.

5) Are Evo-Devo explanations "too internalist"? Evo-Devo goes populational

As Wilkins (1998) rightly pointed out, the explanatory mode in Evo-Devo is resolutely "internalist"; that is, too focused on the specific nature of the genetic alterations involved and paying scarce attention to the selectionist-population dynamics helping to spread such genetic changes. Upstream regions of some genes bear polymorphic regions which may bring major differences at the level of gene expression. Such regulatory polymorphisms in populations are thought to be more abundant than current data would suggest (Rockman and Wray, 2002). Therefore, seeking polymorphisms in regulatory regions of genes for relevant traits would be necessary to overcome such an internalist approach and to explain how crucial genetic changes affecting development spread during evolutionary radiations. To do that, evolutionary developmental biologists should seek and learn the mathematical and modelling skills of population geneticists, whereas the later must devise population genetic models to grasp developmental biology changes.

To start with, it seems wiser to leave aside big hot problems such as the basis of the "Cambrian explosion" (despite their tremendous interest) and concentrate on intra-phyletic, and even better intra-class, intra-order and inter-generic, comparisons. Among the best examples of this approach one could mention the role of *Ultrabithorax* in morphological differences between *Drosophila* species (Stern, 1998), the divergence of cis-regulatory sequences in the *achaete-scute* gene complex between *Drosophila melanogaster* and *D. simulans* (Skaer and Simpson, 2000), the variability among the *even-skipped* stripe 2 in *Drosophila* (Ludwig *et al.,* 2000), differences in structure and function of the *bicoid* gene in different fly species (Shaw *et al.,* 2001) and molecular and cellular variability in vulval development within and among different species of nematodes (Delattre and Félix, 2001). To those, we could add how the gene network has changed during the evolution of parasitic wasps from non-parasitic ancestors (Gbric, 2003) and how some *Hox* cluster genes (e.g. *bicoid*) and segmentation genes (e.g. *hunchback, orthodenticle*) changed their roles in long germ band embryos of higher dipterans (e.g. *Drosophila;* see Lynch and Desplan, 2003), superimposing their activity onto a preexisting regulatory network.

6) Postgenomics and Systems Biology: how do they help Evo-Devo?

The deluge of information on genes, proteins, cellular dynamics and organisms' responses to mutations and the environment, and how to integrate this into a more complete picture of how biological networks from cells to whole organisms function, is the biggest challenge of Biology today. It requires the concerted effort of all kinds of biologists together with mathematicians, bioinformaticians, model builders, computer engeneers and ecologists. The premium is to understand the big misteries of Biology, such as how cells divide and animals develop to how evolution took place.

As regards Evo-Devo, uncovering the structure, functioning and properties of genetic regulatory networks (GRNs) is one of the greatest challenges of the postgenomic era. To do this, biological systems have to be modelled in terms of an information system and the system analyzed mathematically. In recent years, models have become more detailed by incorporating more data in the form of already known and new genes, developmental pathways, regulatory DNA sequences discovered through experiments which disrupt normal gene function, and pinpointing connections between various genes and regulatory regions (Davidson *et al.,* 2003; Hinman *et al.,* 2003; Revilla-i-Domingo and Davidson, 2003). An expected outcome is that as more data is incorporated into the model, more accurately complex biological responses (e.g. changes in gene expression and subsequent changes in cell behaviour) can be predicted. For embryonic development, some general principles are already emerging from GRN architecture (Davidson *et al.,* 2003), namely: 1) that development is moved forward by intergenic positive feedback loops that stabilize, or lock down, a newly set-up regulatory state; and 2) that transcriptional (and/or translational) repression is extensively used to exclude regulatory states set up in one spatial domain from other spatial domains. The final outcome will be to predict *in silico* how systems behave in response to genetic mutations or under specific environmental circumstances. Translated to Evo-Devo problems, it could help to predict the qualitative and quantitative changes in GRN architecture necessary to transform one form or character into another and to make educated guesses as to how evolutionary novelties arose (see below).

Developments to be expected in the future will lead to the establishing of rules for these networks as well as the establishing of sound parallelisms with neural networks, ecological food-webs and social networks (Krause *et al.,* 2003; Neutel *et al.,* 2003). From these studies, general principles of biological networking organization will emerge. In addition, comparative studies of GRNs will also make possible the identification of the architectural GRN motifs common to all bilaterians. This knowledge will, in a not too distant future, help to set the foundations of experimental Evo-Devo (see below).

7) Understanding needs perturbing: towards experimental Evo-Devo

The ultimate goal of any scientific hypothesis or theory is to test, through experimentation, the predictions made from that theory. When applied to Evo-Devo, a previous simple question arises: is Evo-Devo testable? A common practice or test in most recent Evo-Devo studies is to associate differences in the expression levels of

developmental genes with differences in morphological traits (e.g. shifts in axial body regions paralleling axial shifts in *Hox* expression; Burke *et al.,* 1995; Belting *et al.,* 1998). A converse test associates similarities in gene expression and function to a morphological character between distant organisms as a way to prove that their last common ancestor likely had such a character and the genetic toolkit to build it (e.g. similar genetic machinery to make segments must have existed in the last common bilaterian ancestor, or Urbilateria, and parts of it may have been conserved in annelids, arthropods and chordates; Balavoine and Adoutte, 2003).

Interesting as they may seem, these findings are just associations, but not causal findings (Wagner, 2001). To demonstrate a causal role, a developmental genetic difference has to be shown to be the proximal cause, that is, to produce a derived character state. In other words, a genetic change has to be introduced into the genome of a species bearing the ancestral character state and shown to produce the derived character state. This sort of experiment requires genetic manipulation or, even better, building up transgenic organisms. Two examples, the first one close to actual science and the second to wild dreaming, could be contemplated. As pointed out by Wagner (2001), the work of Keys and co-workers on the origin of eye spot patterns in nymphalid butterflies (Keys *et al.,* 1999) represents one of the best understood examples of an evolutionary innovation. Causing loss of *cubitus interruptus* (ci) repression by engrailed in transgenic non-nymphalid (not bearing eyes in the wings) butterflies may induce the appearance of a structure resembling eye spots. If it works, we will be much closer to understanding its causality.

The second example is close to what Evo-Devo fans dream of, although it is more wishful wizardry than actual Science. A popular explanation on the origin of bilaterians is by progenesis from the planula larva of cnidarians (the planuloid-acoeloid hypothesis; see Willmer, 1990, for general references). Planula larvae are symmetrically radial and have a single body axis (Oral-Aboral) and two germ layers (ectoderm and endoderm). There is a growing consensus that the extant bilaterians closest to present day cnidarians are the acoelomorph plathelminthes (Baguñà *et al.,* 2001; Jondelius *et al.,* 2002; Ruiz-Trillo *et al.,* 1999, 2002; Telford *et al.,* 2003). Acoelomorphs are bilateral, with two body axes (A/P and D/V) and three body layers. Besides scores of minor differences, such features represent key derived characters of acoelomorphs compared to the corresponding ancestral characters in cnidarian planulas. Of these features, bilateral symmetry and the ensuing two orthogonal body axes seem to be the true important key innovations. How can the radial symmetry of a planula be broken down? A likely way is to induce (either by activation or repression) the asymmetric expression of an ancestrally expressed gene X radially along the Oral-Aboral axis (for current and potential symmetry-breaking mechanisms, see Meinhardt, 2001, 2002). This might result in two complementary territories defined by two genetic states (X-on; X-off) which may represent an emergent dorso-ventral axis. Studies of gene expression in planula larvae show a preferentially radial expression at the posterior (blastoporal) body end (Finnerty and Martindale, 1999; Hobmayer *et al.,* 2000; Spring *et al.,* 2002; Scholz and Technau, 2003; Yanze *et al.,* 2001; Wikramanayake *et al.,* 2003; though see Hayward *et al.,* 2002, reporting the asymmetric expression of an ortholog to the *dpp/BMP2/4* gene at the blastopore in coral embryos). To induce asymmetry, gene expression should be inhibited locally using interference RNA (RNAi) or Morpholinos. Alternatively, transgenic planulas could be engineered to ectopically express or enhance at one side of the blastopore any of the genes involved in mesoderm formation or setting bilateral symmetry in bilaterians (e.g. *caudal, Forkhead, Brachyury,*..) and observing the effects.

Even if these experiment gave observable, repeatable and meaningful effects, it is impossible to know whether, 600 million years ago, it happened that way. A scientifically sounder way is, once acoelomorphs and cnidarians are beyond doubt shown to be the extant closest sister-groups, to uncover the key elements of their GRNs responsible for radial/bilateral symmetry and endoderm/endomesoderm formation, together with working out a detailed account of their expression patterns and their interrelationships. On such a basis, specific perturbations could experimentally be tested and those leading to symmetry breakings further analyzed. Were all/any of these approaches shown to work, they will in the long run (or maybe never), transform an ancestral character state into a derived one. Whenever this happens, Development and Evolution will change from a descriptive science into an experimental one with very far-reaching consequences.

A final caveat. Evo-Devo experiments can only be carried out with organisms which are currently alive and which bear genotypes with genetic backgrounds that may significantly differ from those in the ancestral population which actually made the transition. In other words, even if the "correct" genetic change is introduced, there may be no effect or the effects may be different to those expected (Wagner, 2001). An even if the expected effect is reproduced, we will never know whether this was how it actually happened, for the simple reason that we have no way of knowing the main features of the genetic background of the ancestor. Despite these important methodological caveats and hindrances, it is preferable and better fun to get on with it than to give up and ignore it.

8) A hot Evo-Devo agenda

Comparative developmental genetics (and developmental genomics) in closely related species

It is becoming clear that evolution in action is easily recognizable in closely related species. Small changes in satellite species compared to well established models is providing nice surprises, e.g. the high level of genetic divergence between two morphologically indistinguisable species of nematodes (Blaxter, 2003), or the remarkable genomic differences between *Drosophila* species and with the mosquito. This approach will give insights into the relevance of species-specific genes (e.g. human, primate, rodent etc.) and into the plasticity of the genome, by identifying genetic changes not translated into morphological differences.

Major macroevolutionary events

The origins and (wherever applicable) radiation of: a) multicellularity, b) body symmetries, c) bilaterian animals, d) the Protostome/Deuterostome split, e) vertebrates and f) land plants, together with whether the "explosion" was a Cambrian or a PreCambrian affair, are among the hottest macroevolutionary events to be dealt with in the future. Developmental genomics, cis-genomics and expressomics of relevant animals at the crossroads of these events are urgently needed to fully understand the structural evolution of the genome and to identify dramatic (and less dramatic) changes concurrent with such key changes. Furthermore,

the development of these animals as functional model systems (to "perturb", see above) will open experimental scenarios in which predicted changes, co-options and recruitements of genes and regulatory networks can be tested.

Redefining/refining homology

Finding that similar (homologous) genetic machineries build divergent phenotypes and, conversely, that different phenotypes can be built using non-homologus gene tools is reshaping our views on what homology is. An extreme view (Hall, 2003) sees all sort of homoplasies, except convergence, as homologous at the genetic level. One foreseable task of Evo-Devo is to set the limits between homology and bricolage, consisting of independent recruitment of a gene network and, in the end, to ascertain at what levels evolutionary constraints favor the recurrent invention of certain features, while preventing others from emerging.

Intrinsic properties of gene regulatory networks (GRNs)

Exploring down to the finest detail the properties of GRNs using experimental and theoretical models is another major Evo-Devo challenge. Such a task will be facilitated by parallel developments in new high-throughput molecular and genetic techniques and increasing computing power. Concepts such as robustness, buffering, evolvability, hierarchy or connectivity will be investigated by altering well established gene networks, both experimentally and *in silico*.

Population developmental genetics

Evo-Devo needs population genetics to explain how crucial genetic changes affecting development spread during evolutionary radiations. We have to explore in depth intra- and inter-specific coding and non-coding polymorphisms of developmental genes in natural populations. Moreover, we have to seek for conserved cis-regulatory sequences highlighting potential functional sites, for linkage desequilibrium uncovering gene clustering and for natural variability of potential for evolutionary drift. Finally, population mathematics needs to be incorporated into Devo and developmental gene variations and models into traditional evolutionary thinking, in order to find a synthetic new synthesis of evolution.

Experimental Evo-Devo

What we call the third wave of Evo-Devo research will test predictions drawn from actual comparative studies, in experimental systems. Some pioneer experiments have revealed that changing key aspects of a gene network results in the appearance of wings or the reduction/loss of legs, mimicking changes that may well have happened in evolution. At present, the experimental approach to Evo-Devo is strictly limited to very few model systems and experimental situations. To put it plainly, in order to demonstrate the causality of the tetraploidization event at the origin of vertebrates, it would make no sense to duplicate twice the genome of amphioxus and wait until the next day to see a tetrapod walking in the lab! Rather, experiments have to be set up to modify in subtle ways gene networks by ectopic mis-sexpression, cis-regulatory changing, gene silencing or knocking-down, in priviledged key model systems. Such model systems should be: 1) those in which such experiments are already technically feasible allowing deep analyses of the main networks; and 2) those Evo-Devo landmark models (selected for their primitiveness, although less fitted technically), which are suitable to verify the effects of gene network recruitment in places or times spotted by Evo-Devo as critical for the deployment of new features.

Last but not least: more fossils please!

Knowledge of the fossil record at the lower depths of the Cambrian and Precambrian has advanced at a striking pace. Even so, *bona fide* fossils of cnidarian embryos and adults of these faunas, together with their potential relationships to the rich Ediacaran assemblages fall short of being adequate. Currently, vague cnidarian-like fossils have been reported from precambrian beds (see Chen *et al.,* 2002 and Conway-Morris, 2003, for recent summaries), but no acoelomorphs have so far been uncovered (likely because of low preservation potential), and fossils from other potentially basal phyla (e.g. gastrotrichs, chaetognaths, basal ecdysozoans) are very scarce. In any event, their richness and preservation of details are ages away from the spectacular display and richness of bilaterian fossil forms occurring 50 million years later at the Cambrian. Records for other big radiations (e.g. vertebrates, land plants, reptiles, mammals), though incomplete at early stages, are rich enough at late stages to trace the step-by-step accumulation of apomorphic changes. As Budd (2001b) persuasively pointed out, to anyone interested in studying how a living group (e.g. a bilaterian) evolved its derived characters and the principal transitions that led to it (e.g. diploblast-triploblast transition; that is, bilateral symmetry, a second body axis, third germ layer and anterior nervous system), living forms are of no help. Only fossils of its stem group can really help; hence, more fossils please!

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