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MOLECULAR PALAEOBIOLOGY

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Abstract: For more than a generation, molecular biology has been used to approach palaeontological problems, and yet only recently have attempts been made to integrate research utilizing the geological and genomic records in uncovering evolutionary history. We codify this approach as Molecular Palaeobiology for which we provide a synthetic framework for studying the interplay among genotype, phenotype and the environment, within the context of deep time. We provide examples of existing studies where molecular and morphological data have been integrated to provide novel insights within each of these variables, and an account of a case study where each variable has been tackled to understand better a single macroevolutionary event: the diversification of metazoan phyla. We show that the promise of this approach extends well beyond research into the evolutionary history of animals and, in particular, we single out plant evolution as the single greatest opportunity waiting to be exploited by molecular palaeobiology. Although most of our examples consider how novel molecular data and techniques have breathed new life into long-standing palaeontological controversies, we argue that this asymmetry in the balance of molecular and morphological evidence is an artefact of the relative 'newness' of molecular data. In particular, palaeontological data provide unique and crucial roles in unravelling evolutionary history given that extinct taxa reveal patterns of character evolution invisible to molecular biology. Finally, we argue that palaeobiologists, rather than molecular biologists, are best placed to exploit the opportunity afforded by molecular palaeobiology, though this will require incorporating the techniques and approaches of molecular biology into their skill-set.

Key words: evolutionary tempo, evolutionary mode, diversity, disparity.

'I LIKE to take the catholic view that palaeontology deals with the history of biosphere and that palaeontologists should use all available sources of information to understand the evolution of life and its effect on the planet. Viewed in this way the current advances being made in the field of molecular biology are as important to present-day palaeontology as studies of comparative anatomy were to Owen and Cuvier … This does not mean that palaeontologists must adopt a passive role as educated observers of this explosion of knowledge. Instead, palaeontologists have the kinds of skills that are required to develop a general understanding of the experimental results that are flooding the literature at the present time. Most molecular biologists have limited training in the classical disciplines of biology and little appreciation of the nature of the fossil record and the dimensions of geological time. Their remarkable experimental and inductive skills will be strengthened through interactions with scientists having an expert knowledge of the history of life and the large-scale processes and effects of evolution.'

This is the introduction to the published version of the 1985 Annual Address of the Palaeontological Association by Bruce Runnegar, and it is his codification of a research agenda for the coming decades: Molecular Palaeontology (Runnegar 1986). As he saw it, palaeontologists, by definition, study the history of the biosphere, and thus should use all available sources of data to understand the origin and evolution of life on Earth. With the explosion of molecular data starting in the late 1960s, Runnegar argued that palaeontologists would be foolhardy to ignore this burgeoning data set, and, of course, he was (as usual) right on the mark. But despite the even greater overlap between the goals of contemporary palaeontology and molecular biology today, as we will show, Runnegars' landmark paper has received only 16 citations over the last 20 years according to ISI's Web of Science (11 February 2007). To rectify this omission from the consciousness of palaeontologists, we take this opportunity to reintroduce this most prescient paper, and to propose a working definition and a research agenda for Molecular Palaeontology for the coming years.

MOLECULAR PALAEONTOLOGY: THEN ... AND NOW

Although Runnegar (1986) argued generally for the integration of molecular biology into palaeontology, he identified three key areas of opportunity: biomarkers (chemical fossils such as hydrocarbons), biomineralization and 'molecular fossils' within modern DNA. How have these fared? The science of biomarkers has matured into a very healthy and productive discipline that interfaces strongly with palaeontology, such as in debates over the emergence of atmospheric oxygen (Summons *et al.* 2006), the rise of eukaryotes (Brocks *et al.* 1999) and the mechanisms of mass extinction (Grice *et al.* 2005), to give just a few examples. However, it has not been readily incorporated into palaeontology as a line of inquiry or a suite of techniques, and it is generally perceived as a branch of biogeochemistry. This may (and should) change as more palaeontologists apply organic geochemical techniques to great effect, such as to the improved understanding of the processes underlying organic preservation (e.g. Briggs 1999; Gupta *et al.* 2006*a–c*).

Runnegar's second proposal was more speculative. He proposed that it might be possible to unravel the evolution of biopolymers in the construction of skeletons. His vision was to exploit the correlation between mineral microstructure and the organic matrix utilized in biomineralization, mainly by identifying crystal forms or arrangements not encountered among inorganic crystals. In large part, a realization of this vision has been precluded by a dearth of understanding of organic-mineral interaction during biomineralization. Indeed, the underlying basis of skeletal development, including biomineralization, has been revealed as too complex, perhaps, to achieve the kind of inferential studies that he envisaged. Nevertheless, significant advances have been made in understanding early skeletal evolution among vertebrates that has fed back to understanding evolution of the molecular underpinnings of its development (Smith and Hall 1990; Donoghue 2002; Donoghue and Sansom 2002; McGonnell and Graham 2002; Kawasaki *et al.* 2004).

However, it was the third area of the proposed synthesis between molecular biology and palaeontology that occupied the majority of his article and it concerned the historical record contained with organismal genomes, which he termed 'molecular fossils'. The idea follows that proposed by Pauling and Zuckerkandl (1962) that informational biopolymers such as nucleic acids and proteins record their own history. In contrast to the first two components, this leg is necessarily indirect, as it focuses on the genomes of living organisms to extract information about the history of life. Because of this, it is often thought to belong within the intellectual domain of molecular biologists, but as we argue throughout this paper, the very nature of the questions addressed using molecular fossils necessitates that its practitioners are schooled in both the direct geological record and the indirect genetic record, because these questions pertain to deep time, are macroevolutionary in nature, and require the integration of these two seemingly disparate records in order to obtain a holistic understanding of evolutionary history.

Molecular fossils

Runnegar's perception of a research agenda based on molecular fossils was one that addresses questions of evolutionary rate and pattern, disciplines that are now recognized as molecular phylogenetics and molecular clocks. Runnegar himself did much to encourage the application of molecular clocks (Runnegar 1982), but both fields became mainstream only with the development and widespread application of molecular cloning and sequencing techniques in the late 1980s (Delsuc *et al.* 2005; Kumar 2005).

The discoveries made on the back of molecular phylogenetics and the attendant application of molecular clocks cannot be overstated. Over a century of debate has been laid to rest concerning the monophyly of Metazoa and the high-level relationships among the component phyla (Eernisse and Peterson 2004; Halanych 2004). For example, brachiopods and bryozoans, which were traditionally classified with the deuterostomes, are now denizens of Lophotrochozoa (Halanych *et al.* 1995), the rump of which is composed of members of the now defunct Articulata that are not members of Ecdysozoa (Aguinaldo *et al.* 1997). All protostomes belong to one or other of these two superclades, while the deuterostomes are composed of just three major phyla, the echinoderms and hemichordates, together comprising Ambulacraria, and the chordates (Smith *et al.* 2004), and one minor phylum, represented only by *Xenoturbella* (Bourlat *et al.* 2003, 2006). Sponges are an extensive paraphyletic grade and not a clade, and acoel flatworms are basal triploblasts (reviewed in Peterson *et al.* 2005). Molecular clock analyses are increasingly sophisticated, attempting to incorporate more realistic constraints on molecular evolution, and achieving divergence times that are converging on palaeontological estimates (see below).

Thus, molecular biology has become integral to questions within the sphere of palaeontology. However, the application of molecular biology that has emerged to be of greatest significance to palaeontological questions is developmental genetics and, in particular, evolutionary developmental biology. Runnegar did not foresee this, not least because the rudiments of a discipline combining evolutionary and developmental biology were barely in

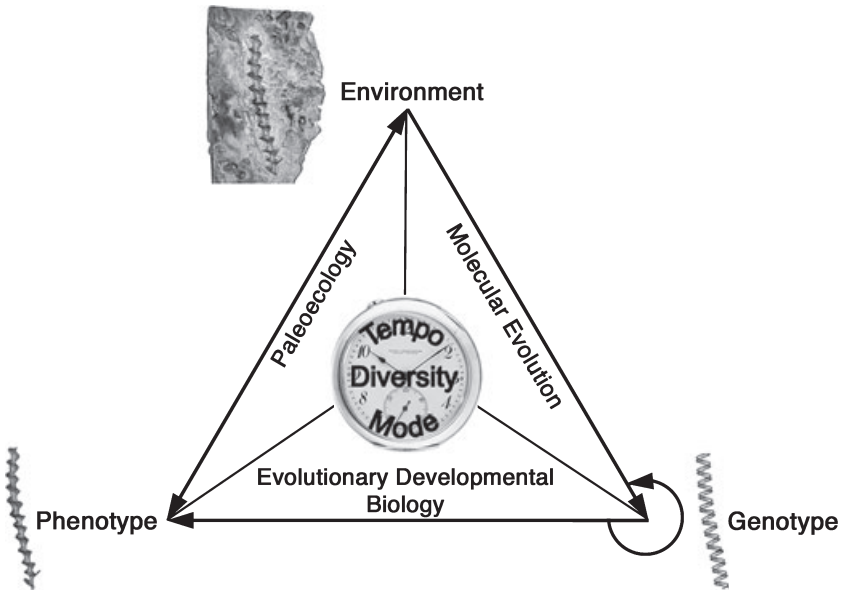
place in 1986. The importance of embryology in evolutionary change has been recognized since the late nineteenth century, but its incorporation into mainstream evolutionary biology was delayed for almost a century, when most embryologists turned away from Haeckelian recapitulation and evolutionary theory to concentrate instead on developmental mechanics (Amundson 2005). Although there were proponents of an evolutionary developmental biology throughout the last century (Love and Raff 2003), its mainstream re-emergence was predicated on the discovery of the genes that regulated development (Lewis 1978; Nüsslein-Volhard and Wieschaus 1980), and the development of techniques that allowed these genes to be cloned, sequenced and compared across a wide range of metazoan taxa (Davidson 1994). The first examples of this were members of the *Hox* family of transcription factor-encoding genes, revealed to possess a conserved homeodomain by all members of the *Antennapedia* class and across all phyla examined (McGinnis *et al.* 1984; Holland and Hogan 1986), conservation that extended to regulatory functions of these genes during development (Carroll 1995). This discovery, and the avalanche that followed, provided for the first time a synthetic and experimental basis for understanding and uncovering the relationships between genes, development, morphology and evolution, and introduced an overlap in interests between developmental geneticists and palaeontologists.

The model organism approach on which the discoveries of *Hox* gene conservation were based naturally led to a renewed interest in the large-scale questions of animal evolutionary history, such as the origin of animal body plans, because the comparisons between developmental data sets were taxonomically so broad (invariably between fly and mouse; e.g. Graham *et al.* 1989). Indeed, the influence upon this debate of developmental genetics has been so pervasive that for many years it has been impossible to evade discussion of the role of regulatory genes in considering the origin of animal phyla. The widespread vicariance of a genetic toolkit of transcription factors and cell-signalling molecules, many of which were implicated as 'master control genes' for the embryonic development of specific anatomical features such as segmentation, heart, limb and eye development (Gehring 1999), led to a refocusing of evolutionary debate from considering why animals are so different to how such disparity can be achieved on the basis of genetic conservation. In addition, the intensive focus upon metazoan model systems has provided an in-depth understanding of how genes direct development and, by linking genes to morphology within a comparative framework, detailed developmental genetic explanations for the origin of many of the key innovations identified classically by palaeontologists, such as the origin of jaws (Takio *et al.* 2004) and the origin of fins (Freitas *et al.* 2006), are now available.

Thus, we are now in a position, both technically and methodologically, not only to explore this molecular fossil record but also to integrate it with the geological fossil record and, hence, to realize fully Runnegar's vision of a holistic science dedicated to studying the evolution of life. This represents a real opportunity for palaeontologists to integrate molecular techniques and approaches with traditional palaeontological questions, and to provide an experimentally based mechanistic understanding of the pattern of evolutionary history revealed by molecular phylogenetics, molecular clocks and the geological record. To promote this holistic approach we propose and codify it as Molecular Palaeobiology.

WHAT IS MOLECULAR PALAEOBIOLOGY?

We envisage Molecular Palaeobiology as a distinct approach within Palaeontology incorporating the materials and methods of molecular biology, and dedicated to understanding both the tempo and the mode of evolution, as well as understanding how diversity (both character and taxic) changes through time, by studying the interface between genotype, phenotype and environment. Perhaps inevitably, because we are palaeontologists (e.g. Seilacher 1970; Seilacher *et al.* 1985) we represent the relationship between these factors in the form of a ternary diagram (Text-fig. 1). Genotype links to phenotype in the process whereby one-dimensional genetic information is translated into three-dimensional morphology. In turn, the phenotypic outcome of the genotype is dependent on environmental and ecological context, in terms of both epigenetic control of development and the filter of natural selection. However, the interplay of these variables also has a temporal perspective and to reflect this, we must add a third dimension. Each of the component sides effectively represents established disciplines: genotype-phenotype-time represents evolutionary developmental biology; phenotype-environment-time represents palaeoecology; and environment-genotype-time represents molecular evolution. The interface of these three subject areas, molecular evolution, evolutionary developmental biology ('evo-devo'), and palaeoecology, is the theme of Molecular Palaeobiology, as it uniquely integrates the patterns written in the two historical records, genomic and geological, to infer both the tempo and the mode of evolution, and to understand the history of diversity, by integrating information from the nature of genetic change, through developmental morphology, and up through ecological developmental biology. This integration would then allow palaeobiologists to address the following sorts of questions:



TEXT-FIG. 1. Molecular Palaeobiology. See text for details.

Tempo

1. How fast does evolution occur, at both the morphological and the molecular levels?
2. What is the relationship, if any, between the rate of change of genotype and the rate of change of phenotype?
3. What is the meaning of significant mismatches between palaeontological and molecular clock estimates for the timing of major evolutionary events?
4. Is the relationship between genomic and epigenetic evolution one-way traffic?

Mode

1. Is morphological and/or molecular evolution a continuous process or does it run in fits and starts?
2. How do changes in genes and gene regulatory networks affect morphology through time?
3. What insight does the empirical record of fossil morphology provide about the evolution of developmental processes?
4. How do novel structures arise? How do novel genes arise? Is the former dependent upon the latter? Does the latter always generate the former?

Diversity

1. How has diversity changed through time? What roles do internal and external influences play in the establishment and subsequent evolution of the modern biota?
2. How has disparity changed through time, and what is the underlying molecular basis for disparity?

Answers, even partial, to any or all of these questions would give tremendous insights into the evolutionary process and will lead to a much deeper understanding of the living biota. Hence, the codification of Molecular Palaeobiology is, in theory, very laudable, but is it pragmatic? In the next section we discuss how molecular data and techniques are already being employed and integrated with palaeontological data and hypotheses to address the kinds of questions that we have set out under the themes of genotype, phenotype, environment, tempo, mode and diversity. After this we provide a case study that integrates all aspects of molecular palaeobiology to provide a more holistic insight into one of the most fundamental events in evolutionary history: the emergence of animal phyla. We then explore opportunities in other areas of Palaeontology, and in particular highlight plant evolution as the single greatest opportunity waiting to be exploited by molecular palaeobiologists. Finally, we explore the unique contributions made by palaeontological data, in particular the importance of the stem group, and argue that only molecular palaeobiologists will have the necessary skill sets to address the sorts of questions posed above.

MOLECULAR PALAEOBIOLOGY: THE HISTORICAL PATTERNS

Genotype

There has been a long and rich tradition in utilizing molecular data to address phylogenetic hypotheses in the context of unravelling the history of life (Pagel 1999), and

nowhere is this more apparent within the palaeontological community than with the phylogenetic position of whales. Their placement within Mammalia was long considered an enigma (Simpson 1945) until Van Valen (1966) placed them with an archaic group of ungulates, the mesonychids, and most palaeontologists have strongly supported Van Valen's taxonomic assignment ever since (reviewed in Gatesy and O'Leary 2001). However, Simpson's (1945) challenge, the placement of Cetacea within Mammalia, was also taken on by molecular biologists. In probably the first molecular palaeobiological study, Boyden and Gemeroy (1950) argued, using serum proteins, that cetaceans exhibited greater similarity to artiodactyls (the even-toed hoofed mammals) than to the other mammalian orders analysed. Subsequent palaeontological discoveries corroborated this view, especially the discovery that stem-cetaceans like *Basilosaurus* had paraxonic feet (paraxony is the possession of an axis of symmetry run between digits three and four), an apomorphy of Artiodactyla (Gingerich *et al.* 1990). Hence, until 1994, the most parsimonious interpretation of the data was that cetaceans and artiodactyls are sister taxa, and mesonychids stem-cetaceans (Gatesy and O'Leary 2001).

The addition of sequence data dramatically changed this perspective. Graur and Higgins (1994), based on a phylogenetic analysis of protein and mitochondrial DNA sequences, argued that cetaceans were not only closely related to artiodactyls but were actually nested within Artiodactyla, making artiodactyls paraphyletic, and Irwin and Arnason (1994) showed that within Artiodactyla, cetaceans were most closely related to hippos than to any other group. Subsequent studies confirmed the paraphyly of Artiodactyla and the sister grouping of cetaceans and hippos (Gatesy *et al.* 1996; Gatesy 1997; Gatesy and O'Leary 2001; Murphy *et al.* 2001). From a palaeontological perspective, this was deeply problematic because it implied either that the dental similarities between mesonychids and cetaceans were convergent or that the double-trochleated astragalus of artiodactyls reversed to the primitive condition in mesonychids, and neither seemed likely. Instead, it seemed that there must be problems with the molecular sequence analyses, possibly owing to the paucity of Recent taxa under molecular investigation, as opposed to the wealth of fossil taxa available for morphological study (discussed in Gatesy and O'Leary 2001).

Definitive evidence for the paraphyly of Artiodactyla and the close relationship between cetaceans and hippos came from a new source of phylogenetic information, unique retroposon insertion events shared between cetaceans and hippos to the exclusion of other artiodactyls (Milinkovitch and Thewissen 1997). Two groups of short interspersed elements (i.e. retroposons that have been amplified and integrated into the genome via retroposition) were present exclusively in the genomes of ruminants, hippos and whales, but not in camels and pigs, nor in any other mammalian group tested (Shimamura *et al.* 1997). Even more striking, given that the position of integration is almost random, was the demonstration that whales and hippos share unique insertion positions of retroposons (Nikaido *et al.* 1999). This topology was entirely consistent with the molecular phylogenetic studies of Graur and Higgins (1994) and Gatesy (1997), among others, refuting the hypothesis that Artiodactyla, as traditionally defined, is monophyletic (Nikaido *et al.* 1999).

As important as these molecular data were to understanding the broad scale pattern of mammal evolution (Springer *et al.* 2004), it still took the discovery of double-trochleated astragali in stem-cetaceans (Gingerich *et al.* 2001; Thewissen *et al.* 2001) to convince most palaeontologists of the accuracy of the molecular tree (Thewissen *et al.* 1998), and that convergence underlies the similarity between the teeth of cetaceans and mesonychids (Nikaido *et al.* 1999). These results highlighted the need for palaeontologists to question basic assumptions about the quality and usage of what was essentially a single and homoplastic data set (Naylor and Adams 2001; Kangas *et al.* 2004), to re-analyse existing morphological and palaeontological data sets (Geisler and Uhen 2003), and to re-analyse the fossil record and phylogenetic relationships of newly relevant taxa (Boisserie 2005; Boisserie *et al.* 2005*a*, *b*). The converse is also true: the apparent strength of the palaeontological data made molecular phylogeneticists scrutinize their existing data and algorithms, and dramatically increase the size of the original data set (Gatesy *et al.* 1999). Ultimately, however, the important lesson was that both the genetic and the geological fossil records converged on the same answer that whales nest within Artiodactyla.

One of the more fruitful avenues for molecular palaeobiological research beyond unravelling the tree of life is the link between genetic innovations and morphological novelties (Muller and Newman 2005), and a particularly relevant palaeobiological example concerns the evolution of metazoan skeletons. For example, in the sea urchin *Strongylocentrotus purpuratus* there are 15 spicule matrix genes, all of which code for proteins associated with the organic matrix of the stereom skeleton (Livingston *et al.* 2006). All of these genes are novel to echinoderms while genes implicated in vertebrate biomineralization, including the SCPP genes (Kawasaki and Weiss 2003), are absent (Sea Urchin Genome Sequencing Consortium 2006). Hence, a clade-specific feature, echinoderm stereom, is underpinned by a clade-specific set of genes that encode spicule matrix proteins, and the fossil record indicates that these novel genes arose sometime between the split from hemichordates and the first appearance of stereom in the fossil record, about 520 million years ago (Bottjer *et al.* 2006).

The more usual route for morphological novelty is not the evolution of novel genes though, but the use of gene recruitment, especially of gene duplicates or paralogues (Conway Morris 2000*a*). Beyond genome duplications, which are relatively uncommon, tandem gene duplications have often been linked with the generation of morphological novelties. For example, the vertebrate SCPP genes arose by a series of gene duplications from *SPARCL1*, itself a duplicate of the pan-bilaterian *SPARC* (Kawasaki *et al.* 2004). *SPARCL1* duplicated multiple times to give rise to the diversity of SCPPs implicated in the development of: (1) dentine/bone, found in tetrapods and some actinopterygians (but could have arisen earlier; Donoghue *et al.* 2006); (2) enamel matrix, taxonomically restricted to tetrapods; and (3) milk caseins and some salivary genes, taxonomically restricted to mammals (Kawasaki and Weiss 2003; Kawasaki *et al.* 2004). These studies suggest how the advent of morphological novelties through geological time can be tied to their underlying genetic innovations, many of which involve the tandem gene duplication of a pre-existing gene (Kawasaki and Weiss 2003, 2006; Kawasaki *et al.* 2004; Donoghue *et al.* 2006).

Finally, the use of ancient DNA has an important role for investigating the interplay among climate change, phylogeography and population genetics over the last 1 myr or so (Chan *et al.* 2005; Orlando *et al.* 2006), and to test phylogenetic relationships between Pleistocene ancestors and presumed living descendants (Orlando *et al.* 2003; Paabo *et al.* 2004; Bunce *et al.* 2005; Kuehn *et al.* 2005). For example, the use of ancient DNA coupled to the fossil record has allowed workers to relate how the gene pool of prehistoric populations changed through time, driven by external factors such as climate change (Shapiro, B. *et al.* 2004; Chan *et al.* 2005) and possibly biotic competition (Barnes *et al.* 2002). Indeed, the field of palaeogenomics, or the study of ancient genomes (Birnbaum *et al.* 2000), has the potential to give unparalleled insights into DNA polymorphisms present in extinct populations, and thus to the population genetics of a taxon over thousands of years and while experiencing dramatic environmental upheavals (Nicholls 2005; Noonan *et al.* 2005; Poinar *et al.* 2006). In addition, the portentous addition of the Neanderthal genome to the list of taxa under palaeogenomic investigation has the potential to shed light on those genomic changes that constitute the core of *Homo sapiens* since its split from *Homo neanderthalensis* roughly 500,000 years ago (Green *et al.* 2006; Noonan *et al.* 2006). Owing to the chemical instability of nucleic acids, though, there is no realistic hope for deep-time applications (i.e. before the Pleistocene) in the foreseeable future (Nicholls 2005), although deep time palaeoproteomics appears to be a real and very exciting possibility (Asara *et al.* 2007; Schweitzer *et al.* 2007).

Phenotype

The study of the form, function and phylogeny of fossils, in comparison with extant organisms, is the traditional domain of palaeontology, and much of our knowledge about the history of life on Earth comes from this field. Nonetheless, the molecular and phylogenetic revolutions have provided novel opportunities for investigation, breathing new life into stale debates. We take as our principal example the evolution of the bird manus.

Despite the wealth of fossil evidence to the contrary (Padian and Chiappe 1998), some workers continue to assert that birds are not descended from theropod (i.e. carnivorous) dinosaurs, and the only real evidence to support this assertion centres around the development of the avian hand (Zhou 2004). Developmental biologists have long identified the three digits of the avian hand as 2-3-4, with digits 1 and 5 lost [by comparison to humans, the numbering system reflects the order from thumb (1) to little finger (5)]. This is because in most tetrapods the primary axis is observed to run through the ulna into digit 4 and, thus, if a similar pattern exists in living birds (Burke and Feduccia 1997), the most posterior digit must be 4, the other two being 2 and 3. Theropod dinosaurs, on the other 'hand', are 1-2-3 with the loss of digits 4 and 5 clearly documented in their fossil record (Chatterjee 1997). If, in fact, the manual digits of modern birds are 2-3-4, and theropod dinosaurs are 1-2-3, and without some alternative mechanism (Wagner and Gauthier 1999), then the theropod origins of modern birds would be refuted (Hinchliffe 1997). However, the identification of digit 4 in the bird hand assumes that birds are not descended from theropod dinosaurs, as they assume that like most other tetrapods the first digit to form is digit 4, the very hypothesis they are testing (Thomas and Garner 1998).

Vargas and Fallon (2005*a*) used an independent data set to test the hypothesis that the bird hand is actually 1-2-3, and not 2-3-4 as proposed by Burke and Feduccia (1997). Specifically, in both the mouse and in the chicken foot, digit 1 expresses *Hox13* but not *Hox12*, whereas all other digits express both genes. In the bird hand, the first digit, like digit 1 in other tetrapods, expresses *Hox13* but does not express *Hox12*, and the second digit expresses both (Vargas and Fallon 2005*b*). If the bird manus was actually 2-3-4, then digit 2 should express both *Hox* genes. As it expresses only *Hox13*, the 2-3-4 hypothesis is refuted (Vargas and Fallon 2005*b*). Thus, these data are entirely consistent with a theropod ancestry of modern birds, opening up an unprecedented avenue for molecular palaeobiologists to explore the evolutionary history of this remarkable organ in the greatest of detail given that the pattern of digit loss in both morphological space and geological time is effectively known. Now what needs to be added to the pic-

ture is how digit identity is wired into the tetrapod genome, and how aspects of this gene regulatory network can change over time. Given the remarkable insights already gained into the development and evolution of digits (Zakany *et al.* 1997; Zakany and Duboule 1999; Laurin *et al.* 2000), a 'full' description of the evolution of the dinosaur hand could be right around the corner.

Environment

The third side of our evolutionary prism is the environment and it is here where ecology meets genetics and morphology. We have little to offer here by way of synthesis because palaeoecology has been long dormant within palaeontology, and because considerations of the role environmental factors play in molecular and developmental biology has been deeply unfashionable. However, the situation is changing rapidly on both fronts. The emergence of macroecology as a discipline in the biological sciences (Brown and Maurer 1989; Gaston and Blackburn 2000) holds great potential for application to the Deep Time record and its presence has already begun to be felt in palaeontological debates, such as the emergence of metazoans (e.g. Butterfield 2007). In developmental biology, too, the study of environmental effects upon developmental programmes, resulting in phenotypic plasticity, is beginning to provide an intriguing fifth conceptual dimension to phenotypic evolution (West Eberhard 2003; Hall *et al.* 2004; Pigliucci and Preston 2004). Thus, the environment is likely to be an area of much synthetic development over the coming years, offering great potential for new research programmes. Indeed, Jablonski's (2005; see also Jablonski and Bottjer 1991) onshore-offshore pattern of post-Palaeozoic morphological novelties is of some interest, assuming that it is not a taphonomic artefact (Smith 1994), and highlights the need for a molecular underpinning to these higher-level morphological novelties so that potential hierarchical palaeontological patterns can be grounded into a mechanistic foundation.

MOLECULAR PALAEOBIOLOGY: THE EVOLUTIONARY PROCESSES

What makes Molecular Palaeobiology a distinct branch of palaeontology is not an exclusive focus on patterns, as interesting as they may be, but instead on the evolutionary process as reconstructed by the confluence of these patterns. Three patterns in particular that emerge from the confluence of genotype, phenotype and environment are tempo, mode and diversity.

Tempo

The traditional interface between palaeontology with molecular biology has concerned evolutionary rates. Before the molecular biology revolution, geneticists relied on palaeontologists to understand the rate of evolution (Simpson 1944), which is to be expected given that the primary source of information about evolutionary rates is derived from the fossil record itself (Stanley 1985). Since the molecular revolution, palaeontologists have largely handed this critical area of research over to molecular biologists. Perhaps this outcome is to be expected given that changes in morphology must be underwritten by changes in DNA (Schopf *et al.* 1975; Schopf 1984). Nonetheless, there is clearly a need for interface between molecular evolutionists and palaeontologists, and nowhere is this more apparent than in the use of molecular clocks (Reisz and Muller 2004; Benton and Donoghue 2007).

Molecular clocks require that the rate of molecular evolution be disassociated from the rate of morphological evolution, and many early studies clearly showed a discordance between the rate of morphological change and the rate of molecular change: morphologically static taxa like horseshoe crabs (Selander *et al.* 1970; Avise *et al.* 1994) and frogs (Wallace *et al.* 1971; Cherry *et al.* 1977) showed relatively normal levels of genetic variation, and morphologically plastic taxa like humans showed dramatic molecular similarity with near relatives (King and Wilson 1975; Chimpanzee Sequencing and Analysis Consortium 2005) (reviewed in Wilson *et al.* 1977; Schopf 1984). Many more recent studies have shown similar results, demonstrating the disjoint between morphological and molecular rates of evolution (Cunningham *et al.* 1992; Sturmbauer and Meyer 1992; Smith *et al.* 1995; King and Hanner 1998; Jarman and Elliott 2000; Rocha-Olivares *et al.* 2001). This is not surprising given that most molecular studies focus on ribosomal, mitochondrial and housekeeping sequences, genes whose role in morphological evolution is far from apparent (Schopf 1984). Hence, using molecules such as these to infer divergence times with a molecular clock seems sound in principle, as the rate of molecular change would not be affected by changes in the rate of morphological evolution. Nonetheless, evidence has been presented in support of the contrary view, namely that morphological and molecular evolution are coupled, effectively debasing the molecular clock (Omland 1997). Although this single study was seized upon as a means of refuting molecular clock estimates that are grossly out of accord with the fossil record (e.g. Conway Morris 1998*a*, *b*, 1999, 2000*b*; Lee 1998), it was subsequently shown that there was no correlation between the rate of molecular evolution of these genes and the rate of morphological evolution (Bromham *et al.* 2002).

Thus, in theory at least, the molecular clock is a viable analytical tool, but it still remains an open question whether in practice molecular clocks accurately estimate divergence times (Bromham and Penny 2003). Benton and Ayala (2003) claimed that molecular clock methods consistently overestimate divergence times. However, because many molecular clock studies focus on groups with an inherently poor fossil record (e.g. bird/mammal ordinal diversifications, metazoan phylum-level divergences) it has been difficult to evaluate their claim (but see below for metazoan phyla origins). Smith *et al.* (2006) used the fossil record of echinoids to test among a variety of molecular clock techniques. They constructed phylogenies from both morphological characters and molecular sequences (three ribosomal genes), and importantly showed that each are suboptimal estimates of the others. The combined tree, when calibrated against the fossil record, thus provides minimum estimates for divergence times, and an estimate for the completeness of the echinoid fossil record. As expected, the fossil record is fairly complete at the family level. Interestingly, there is general concordance between the fossil record and the estimates derived from the molecular clock when using certain algorithms under certain conditions (Smith *et al.* 2006). There were only three areas on the tree that showed significant discordance between the fossil record and the molecular clock, and in each case a different reason might explain the discrepancy. In the first case, that of the origin of euechinoids, there is ambiguity surrounding the phylogeny. In the second case, peculiarities in the diagnosis of camarodonts (a group of globulose urchins), which is primarily based on the structure of non-fossilisable pedicellaria, may explain the discrepancy. In the third case, that of clypeasters (sand dollars), there might be a substantial rate increase of the genes studied in these taxa, giving spuriously deep estimates of divergence. Hence, in each case a different reason underlies the discrepancy. The deeper message though is the remarkable concordance over much of the echinoid tree, suggesting that if sequences are modelled properly, a molecular clock can return accurate divergence estimates.

Nonetheless, there must be some correlation between changes in genotype and changes in phenotype, given that the latter ultimately depend upon the former (Text-fig. 1). One would suspect that any correlation (if not causality) cannot lie within the standard phylogenetic markers (Bromham *et al.* 2002), but instead should be found in genes whose products are directly relevant for the development of form (cf. Lee *et al.* 2006). In easily the most famous example, Lewis (1978) argued that the evolution of arthropod segments was a result of the evolution of the homeotic genes of the Bithorax complex. In this landmark paper, Lewis showed that at least eight genetic mutations affected the development of the nine most posterior segments in the fly, the third thoracic segment and the eight abdominal segments (reviewed in Maeda and Karch 2006). Lewis (1951, 1978) proposed that during arthropod evolution, new homeotic genes arose via gene duplication that allowed for the generation of new morphological segments, and thus in the evolution of insects from 'millipede-like ancestors' segmental individuality evolved from segmental homonymy by the addition of new homeotic genes in the Bithorax complex. Therefore, the rate of morphological evolution, in this case segmental tagmosis, was controlled by the rate and fixation of homeotic gene duplications.

Following the Lewis (1978) paper it was determined that the *Bithorax* complex did not consist of 8–9 genes, but three: *Ubx*, *abd-A* and *Abd-B* (Martin *et al.* 1995; reviewed in Maeda and Karch 2006). Instead, nine distinct regions of *non-coding* DNA control the expression of these three *Hox* genes, and these nine regions were found to be the source of the mutants studied by Lewis (Maeda and Karch 2006). This realization harks back to Schopf's (1984) review where he divided the genome into two domains. Type 1 DNA is the genic or the coding portion of the genome, whereas Type 2 DNA is the non-genic or non-coding portion of the genome. Schopf (1984) argued that because Type 2 DNA is present in a far higher percentage than Type 1, and changes far more rapidly, Type 2 DNA is what governs the rate of molecular evolution, and hence controls the rate of morphological evolution. For example, in humans less than 2 per cent of the total DNA codes for protein sequences (reviewed in Frith *et al.* 2005). So what is the remaining *c.* 98 per cent of the genome doing? Of course, a significant fraction consists of regulatory DNA, like that studied by Lewis (1978), and surely plays a significant role in controlling the tempo and mode of morphological evolution (Davidson and Erwin 2006). But the most amazing discovery in the last few years is that in humans (which are not atypical) 57 per cent of the genome is *transcribed* but not translated (Frith *et al.* 2005), resulting in over a billion nucleotides that are encoded into RNA but are not translated into protein. The scope of this phenomenon is staggering: the FANTOM consortium (2005) found that in the mouse there are at least 181,000 independent transcripts (remembering that there are only about 20,000 protein-coding genes), of which half consists of non-coding RNA (reviewed in Mattick and Makunin 2005; Willingham and Gingeras 2006). Hence, a tremendous amount of the genome appears to encode antisense RNA and regulatory RNA molecules whose function(s), for the most part, remain mysterious (Mattick 2005; Michalak 2006; Carninci and Hayashizaki 2007).

The idea that regulatory RNA is important for gene regulation has its roots in the ideas of Britten and David-

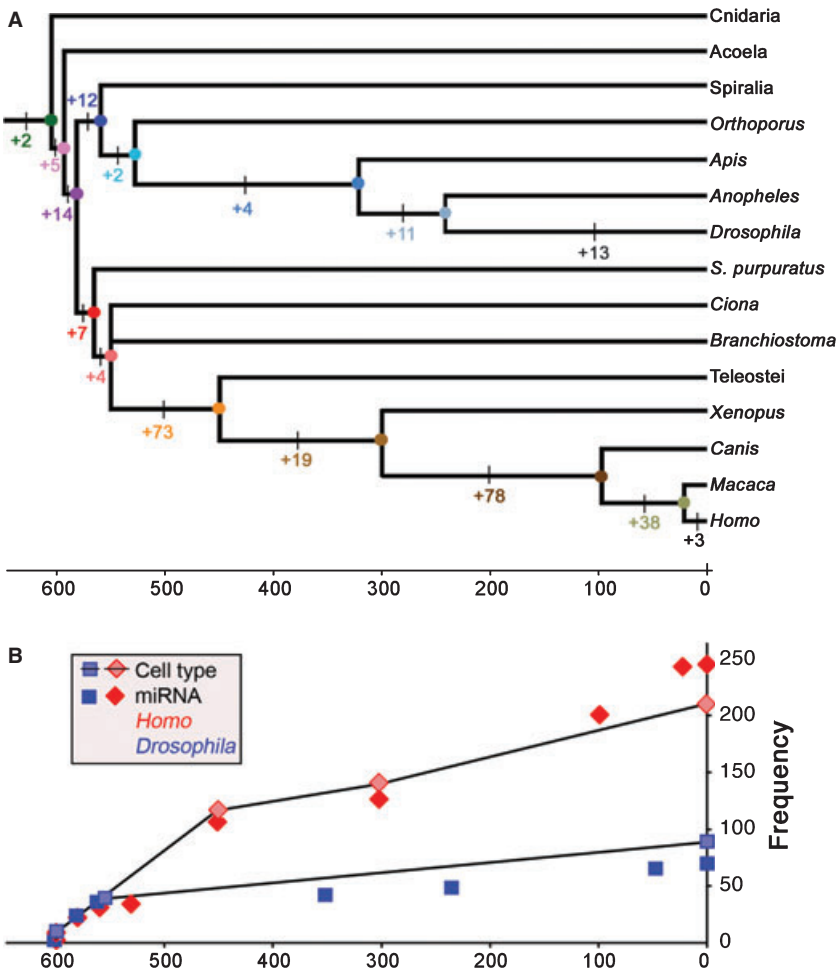
son (1969), but has received a new lease of life with the discovery of microRNAs (miRNAs). miRNAs were first described in 1993 with the report that the gene *lin-4* regulated the timing of cell division in the larval stages of the nematode worm *Caenorhabditis elegans* by negatively regulating the gene *lin-14*. It was determined that *lin-4* encodes a small *c.* 22-nt RNA molecule that binds to sites in the 3′ untranslated region of *lin-14*, which prevents translation of the *lin-14* mRNA (Lee *et al.* 1993; Wightman *et al.* 1993). Small RNA regulation, like that of *lin-4* upon *lin-14*, could have portentous implications for metazoan gene regulation, if it could be shown that it was not simply a quirk of *C. elegans* development (Wickens and Takayama 1994; Lee *et al.* 2004; Ruvkun *et al.* 2004). This would happen with the discovery of a second regulatory RNA gene *let-7* (Reinhart *et al.* 2000), which unlike *lin-4*, was present in many different animal groups and in each seemed to regulate larval to adult transitions (Pasquinelli *et al.* 2000; see commentary by Ruvkun *et al.* 2004). Three research groups then reported the occurrence of many different 22-nt RNA molecules in flies, worms and vertebrates, and christened them miRNAs (Lagos-Quintana *et al.* 2001; Lau *et al.* 2001; Lee and Ambros 2001; reviewed most recently by Plasterk 2006). Largely because *let-7* is absent in all non-metazoans and could not be detected in basal animals like sponges, ctenophores, cnidarians and acoel flatworms (Pasquinelli *et al.* 2000, 2003), Sempere *et al.* (2006) set out to trace the evolutionary history of all 78 *Drosophila* and 313 human miRNAs registered at that time in a variety of taxa, and discovered that the tempo of miRNA gene acquisition mirrors the tempo of cell-type acquisition (Text-fig. 2), which can be used as a proxy for the evolution of morphological complexity (Valentine *et al.* 1994). Therefore, the acquisition of novel RNA regulatory molecules might control to some degree the tempo of morphological evolution through geological time (Sempere *et al.* 2006; reviewed in Niwa and Slack 2007).

And miRNAs might just be the tip of the iceberg (Mattick and Makunin 2005; Michalak 2006). Recently, several new types of RNA coding genes have been discovered in mammals including *HAR1F* (*H*uman *A*ccelerated *R*egion *1 F*orward) (Pollard *et al.* 2006). This genetic locus (called *HAR1*) is remarkably conserved throughout amniotes, but not detected in teleosts or frogs, and dramatically different in humans with 18 substitutions in the human lineage (vs. two between chicken and chimp); these mutations are fixed in our species and have occurred sometime in the human evolutionary line >1 myr. Remarkably, this novel RNA gene is expressed in the human neocortex during the time that cortical neurones are specified and undergo migration, and although also expressed in the cerebral cortex of the monkey, the 18 substitutions suggest that its regulatory role in the developing human brain is different in comparison with all other amniotes. Thus, as Schopf (1984) predicted, and despite the exception that proves the rule (Enard *et al.* 2002), protein evolution may not be a major contributor to the evolution of human apomorphies (Li and Saunders 2005). It remains to be determined if the morphological stasis seen in taxa like horseshoe crabs is owing to the lack of RNA complexity (Schopf's developmental hypothesis) and/or a decreased rate in speciation, as is argued for taxa such as lungfish (Eldredge and Gould's speciation hypothesis; see Gould 2002). Nonetheless, the modern 'RNA world' that is the metazoan genome (Eddy 2001; Bompfünewerer *et al.* 2005) has much to teach us about what might determine, at least in part, the tempo of metazoan morphological evolution.

Mode

One of the longest running debates in evolutionary biology is between those who prioritize form over function, and those who prioritize function over form (Darwin 1859; Russell 1916; Appel 1987; Gould 2002; Amundson 2005). Gould (2002) gave special emphasis to the fact that evo-devo is uniquely suited to testing hypotheses concerning form vs. function. 'Unity of type' or form arguments often focus on the notion of constraints, and hence predict that parallelisms best explain the independent obtainment of morphology in closely related taxa; on the other hand, 'condition of existence' or function arguments prioritize selection, and hence predict that convergence is the underlying reason for morphological similarity (Raff 1996; Amundson 2005; Brakefield 2006). In other words, convergence reflects the functional operation of natural selection upon two substrates different enough to exclude internal factors as influences upon the resulting similarity, whereas parallelism marks the formal influence of internal constraints (i.e. the independent product of 'homologous generators'). The null hypothesis of neo-Darwinism is that phenotypic convergence reflects unique, lineage-specific solutions to evolutionary problems (i.e. convergence). However, the repeated, but phylogenetically independent acquisition of similar character suites within a single clade, which are absent or rare in sister and outgroup clades, refutes the null hypothesis and suggests that the variation presented to selection is not random (Raff 1996).

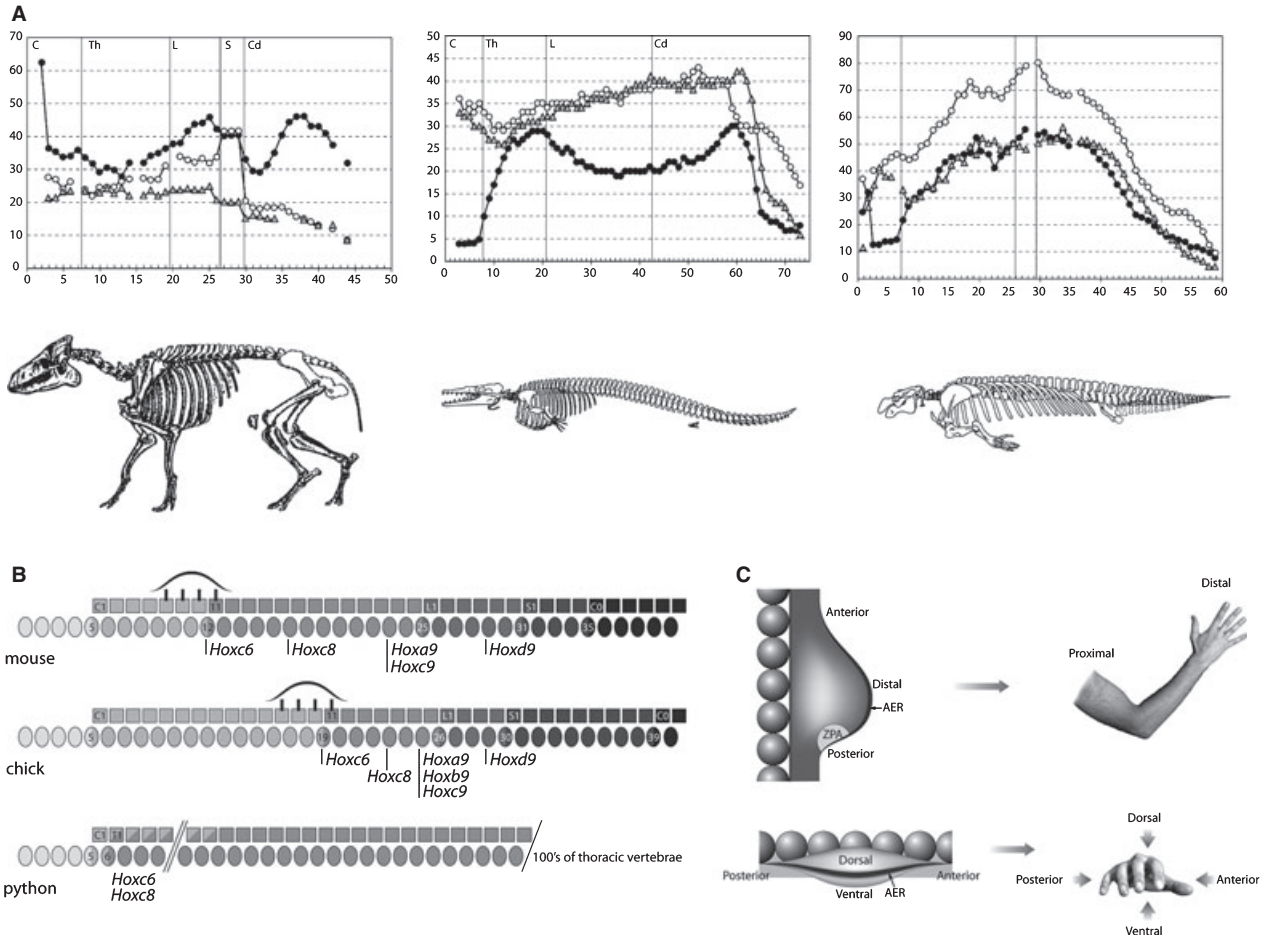
Mayr (1963) predicted that homologous genes would not be found except in very closely related taxa because, as explicated by Gould (2002), adaptive evolution would have crafted and recrafted every genetic locus (if not every nucleotide position) time and time again to meet the constantly changing requirements of continually varying environments. The remarkable conservation of



TEXT-FIG. 2. The evolutionary history of human and fly microRNAs. A, phylogenetic tree of all the taxa considered by Sempere *et al.* (2006) with the relative changes to the 243 human and the 70 fruitfly miRNAs indicated at each node. Note the continuous acquisition of microRNAs in both the fly and the human lineage. Divergence times are taken from both considerations of the fossil record and a molecular clock. Colour code: dark green, Eumetazoa; dark pink, Triploblastica; dark purple, Nephrozoa; red, Deuterostomia; light pink, Chordata; orange, Osteichthyes; light brown, Tetrapoda; dark brown, Eutheria; light green, Hominidae; dark blue, Protostomia; aqua, Arthropoda; light blue, Insecta; lilac, Diptera. B, estimated cell-type numbers for basal members of arthropods and vertebrates, inferred from cell counts of living animals, plotted against time for origin of the respective clades (from Valentine *et al.* 1994). Also shown are the numbers of miRNAs for the *D. melanogaster* and *H. sapiens* lineages over time. From Sempere *et al.* (2006).

Hox transcription factors (McGinnis *et al.* 1984; Holland and Hogan 1986) clearly showed that at least some genetic sequences were under very strong stabilizing selection. Even more remarkable was the demonstration by Averof and Patel (1997) that the independent acquisition of feeding maxillipeds instead of walking legs in several different crustacean groups is a result of the same underlying genetic reason: the independent reduction of *Ubx/AbdA* (*UbdA*) expression in those thoracic segments that primitively express *UbdA* and also construct a walking leg. Other examples include the independent reduction/loss of pelvic fins in different populations of three-spine and nine-spine sticklebacks owing to the loss of *Pitx1* expression in the pelvic fin bud (Shapiro, M.D. *et al.* 2004; Shapiro *et al.* 2006), and the independent recruitment of enzymes into lens crystallins in many different groups of animals (Piatigorsky 1992, 2006; Cvekl and Piatigorsky 1996; Piatigorsky *et al.* 2001) with the addition in each case of *Pax6* binding sites in the promoters of these genes (Cvekl and Piatigorsky 1996; Carosa *et al.* 2002; Kozmik *et al.* 2003). Thus, parallelisms might be much more common than originally thought by the architects of the neo-Darwinian synthesis (Conway Morris 2003).

Because it is intimately coupled to the fossil record, molecular palaeobiology has a unique contribution to this debate because the origins of morphological novelties within a proper phylogenetic context and their stability, if any, over geological time can be studied and eventually understood. A paradigmatic example is the origin of the cetacean body plan as compared with the body plan of sirenians (manatees and sea cows). The use of molecules to test phylogenetic relationships has revolutionized our understanding of mammalian relationships (see above), and large-scale molecular studies have clearly shown that eutherian mammals can be grouped into four monophyletic taxa, Afrotheria (elephants, sirenians), Xenarthra (sloths, anteaters, armadillos), Euarchontoglires (primates, rodents, bats, rabbits) and Laurasiatheria (carnivores, perissodactyls, cetartiodactyls) (Springer *et al.* 2004). The resulting topology showed that some of the traditional mammalian groups (e.g. Ungulata) were polyphyletic, revealing striking morphological homplasy between afrotherians and laurasiatherians (Madsen *et al.* 2001; Sprin-



TEXT-FIG. 3. Parallelism and convergence in the evolution of cetaceans and sirenians. A, centrum length (filled circles), width (open circles) and height (open triangles) of *Pachyaena offifraga* (left), *Delphinus delphis* (dolphin, middle) and *Dugong dugon* (dugong, right) (redrawn from Buchholtz 1998). Shown below (from left to right) are the reconstructed skeletons of *Elomeryx armatus*, *Dorudon atrox* and *Dusisiren* (redrawn from Gingerich 2003; Carroll 1988). Note the similarity between the cetacean and sirenian graphs, especially the shortened neck and loss of a distinctive sacral region, as compared with the primitive mammalian condition (as represented by *Pachyaena*). B, *Hox* gene expression in mouse (top), chicken (middle) and python (bottom). In both the mouse and the chicken the anterior extent of *HoxC6* corresponds to the location of the forelimb; the somites anterior of the limb bud develop in the neck. In contrast, in the python the expression of *HoxC6* extends right up to the atlas (C1): there is no neck and no forelimb. Redrawn from Gilbert (2000). C, development of the vertebrate limb bud showing the positions of the axes with respect to the human arm. Note the position of the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA). See text for details. Redrawn from Logan (2003).

ger *et al.* 2004). Although never considered a clade, cetaceans and sirenians present a potentially fascinating example of parallel evolution: both groups independently acquired an aquatic habitat, and associated with this habitat shift were several remarkable morphological transformations, including the loss of the hindlimb and the homogenization of the vertebrae, as well as a dramatic shortening of the neck. The comparative history of cetacean and sirenian body plan evolution, especially when compared with snakes, is extremely revealing concerning the mode of evolutionary trajectories.

Both cetaceans and sirenians are characterized by exceedingly short cervical vertebrae, and trunks composed of numerous nearly identical vertebrae with short centrum lengths and limited intervertebral mobility (Buchholtz 1998). A short neck both enhances the hydrodynamic shape of the body and stabilizes the anterior end by reducing neck flexibility while the posterior end generates the propulsive force, and only mammals with obligate aquatic habits have cervical vertebrae with centra smaller in length than height (Buchholtz 1998; see Text-fig. 3A). Early whales, such as *Rodhocetus* (Gingerich *et al.* 1994), show foreshortened cervicals, and the modern cervical morphology is seen in more crownward whales like *Basilosaurus* (Buchholtz 1998). Within Sirenia, the basal taxon *Pezosiren* has cervical vertebrae that resemble typical terrestrial mammal propor-

tions and does not show the foreshortened cervicals found in living taxa (Domning 2001).

The question arises as to why both sirenians and cetaceans would reduce the length of the centra rather than lose the cervical vertebrae themselves. One potential answer comes from data obtained from snakes, which have lost all but the first cervical vertebra. Importantly though, snakes have also lost their forelimb and never develop a forelimb rudiment. Studies in tetrapods have shown that the anterior expression of *Hox* genes, specifically *HoxC6*, correlates with the position of the forelimb and serves as a morphological marker for the cervical-thorax transition (Burke *et al.* 1995) (Text-fig. 3B). In the python, transcripts of *HoxC6*, as well as two other thorax markers, *HoxC8* and *HoxB5*, extend right up to the atlas, suggesting that both the transformation of the entire snake trunk into thorax identity and the loss of the forelimbs may be due to the same mechanism, namely the uniformity of *Hox* gene expression along the flank of the snake (Cohn and Tickle 1999) (Text-fig. 3B). Consistent with this suggestion is the correspondence between the two phenotypes with the most basal snake *Najash* possessing both uniform presacral vertebrae morphology and complete absence of forelimbs, but presence of a sacrum and hindlimbs (Apesteguia and Zaher 2006). These data suggest that tetrapods cannot have both a forelimb and lose the cervical vertebrae because there is a genetic constraint such that cervical vertebrae form only anterior to *HoxC6* expression, which is necessary for proper positioning of the forelimb bud. Hence, the only way cetaceans and sirenians could lose the cervical vertebrae would be also to lose their forelimbs, a rather unlikely evolutionary scenario given the important role the forelimbs play in stabilization and steering (Bejder and Hall 2002). Alternatively, they could lose individual cervical vertebrae, but this does not appear to be possible for most mammals (the manatee is an interesting exception with six cervical vertebrae) owing to an unknown, but clearly important, set of genetic/developmental constraints (Galis and Metz 2004; Galis *et al.* 2007). Thus, the only alternative is to minimize the length of the cervical vertebrae in order to shorten the entire neck region.

Limb loss, particularly of the pelvic limbs, is often associated with trunk elongation and axial undulation, and the pelvic appendages of cetaceans, sirenians and snakes are no exception (Carroll 1988; Bejder and Hall 2002). Both cetaceans and sirenians are characterized by the lack of morphological distinction between lumbar, sacral and anterior caudal vertebrae (Buchholtz 1998; Text-fig. 3A), which is again first seen in cetaceans in basal taxa like *Rodhocetus* (Gingerich *et al.* 2001). Importantly, posterior *Hox* genes influence the development of both the limbs and the axial vertebrae. In an elegant series of gene knock-out experiments Wellik and Capecchi (2003) showed that Hox10 genes play a role in suppressing thoracic rib formation, and Hox11 genes play a role in suppressing Hox10 genes, allowing the development of sacral vertebrae as distinct from both lumbar and caudal vertebrae. Thus, the loss of the sacral region in cetaceans and sirenians could have involved the gradual reduction in Hox11 activity resulting in high Hox10 activity from the posterior of the thorax into the caudal series of vertebrae. In contrast, because snakes homogenize their vertebral column by converting lumbar and caudal vertebrae into thoracic vertebrate, their vertebral column might develop without the input of Hox10, rather than Hox11, genes, a clear case of evolutionary convergence.

Importantly, both Hox10 and Hox11 affect the development of the forelimb and hindlimb: Hox10 genes pattern proximal (stylopod) and medial (zeugopod) elements, and Hox11 genes pattern medial and distal (autopod) elements. Thus, reduction of Hox11 activity could result in both the gradual loss of the sacrum and the gradual reduction in limb size by reducing the proliferative rate of limb mesenchyme. Not surprisingly, this cannot be the entire explanation for the homogenization of the cetacean and sirenian vertebral columns because in the mouse elimination of either Hox10 or Hox11 genes does not change the number of vertebrae, only their morphology, so something else must be playing a role in the evolution of these body plans. One possibility is changing the 'clock' mechanism governing somite generation so that more vertebrae develop, which is known to be mechanistically distinct from regionalization and thus could be tinkered with independent of any patterning mechanisms (reviewed and discussed in the context of cetacean evolution in Richardson *et al.* 2004). These data suggest that modifications to the sacrum will inexorably result in changes to the size of the limbs, and thus the body plans of both cetaceans and sirenians might be governed by the pleiotropic constraints of *Hox* genes.

These data and observations predict that cetaceans and sirenians will show modifications of the Hox11 expression patterns, independent of any subsequent or downstream changes to the development of the limb proper. In fact, in both taxa, as well as the python, the loss of the hind limb might be a result of evolutionary convergence. Limbs arise as outgrowths of the body wall, called limb buds, from specified areas along the trunk (Cohn *et al.* 1997). Each limb bud forms what is called an apical ectodermal ridge (AER), which is necessary for proper outgrowth and patterning of the limb (see Johnson and Tabin 1997 for a review of vertebrate limb development, and Text-fig. 3C). Because particular signalling ligands are expressed in the AER, a

second zone of proliferation located on the posterior side of the limb is set up and maintained, called the zone of polarizing activity (ZPA). The ZPA expresses a different signalling ligand from the AER called *Sonic hedgehog* (*Shh*), which is responsible for both the polarity of the limb and the outgrowth of the limb ectoderm: mice homozygous for *Shh* lose most of their distal elements (Kraus *et al.* 2001), whereas ectopic expression of *Shh* on the anterior side of the limb results in mirror-image duplications of digits (Riddle *et al.* 1993). The ZPA is also responsible for maintaining the AER, and thus a series of complicated feedback loops are established in the developing limb bud involving three distinct signalling centres, the AER, the ZPA and the limb ectoderm (Text-fig. 3C).

In snakes, the pelvic limb buds fail to form an AER and do not express AER markers including the signalling ligand *FGF2* and the transcription factors *Msx* and *Dlx* (Cohn and Tickle 1999). In the dolphin, the pelvic limb buds initiate AER formation, and express *FGF* molecules, but a distinct AER is not maintained (Sedmera *et al.* 1997; Thewissen *et al.* 2006). This lack of AER maintenance suggests that a ZPA is not formed and, as expected, Shh is not detected in the posterior limb compartment (Thewissen *et al.* 2006). Thewissen *et al.* (2006) further showed that a second molecule is not detected in the pelvic limb bud, Hand2, a transcription factor that is upstream of *Shh* (Charite *et al.* 2000). However, as they noted, loss of the ZPA probably did not drive the initial loss of the hindlimb because the fossil record clearly shows that basal stem-whales had a fully formed, but slightly smaller hindlimb (e.g. *Rodhocetus*; Gingerich *et al.* 2001; reviewed in Thewissen and Williams 2002). Furthermore, even the most crownward stem-whales, such as the basilosaurids, had distal elements associated with their exceedingly small hindlimbs (Gingerich *et al.* 1990), much more than is seen in *Shh*-mutant mice (Kraus *et al.* 2001). Hence, hindlimb reduction in whales initially involved just the overall size of the limb, not the patterning. Finally, in the sirenians, morphological data suggest that like sticklebacks (Shapiro, M. D. *et al.* 2004; Shapiro *et al.* 2006), but unlike cetaceans, the absence of hindlimbs is because of the absence of *Pitx1* activity, presumably due to mutations in the promoter of the *Pitx1* gene (Shapiro *et al.* 2006).

Thus, the eventual loss of the pelvic limbs in all three taxa appears to be a case of convergence, as they show very different perturbations to hindlimb development, but the initial reduction in limb size in cetaceans and sirenians could be a result of parallelism. These studies highlight the interesting interplay between parallelisms and convergences that have governed the evolution of a body plan, an approach eminently feasible to unravelling the history of body plan evolution in other taxa, and is thus an area ripe for molecular palaeobiological investigation.

Diversity

Understanding what generates biodiversity, and how biodiversity changes over time, is one of the most salient study areas for Molecular Palaeobiology over the next few decades. For example, using a combination of molecular phylogenies and fossil data, Jacobs *et al.* (2004) have shown that the diverse marine fauna of the California coast is not a product of present conditions but is instead derived, in part, from a spike in upwelling and productivity that occurred during the late Miocene. This upwelling regime is a consequence of refrigeration of the deeper ocean that was initiated by the expansion of glaciers on Antarctica. This synthesis links radiation of marine faunas to global-scale climatic changes that have reorganized oceanographic circulation. Similar synthetic analyses are possible on scales ranging from within species to aspects of the radiation of regional and global faunas on both the land and the sea. However, to date, even at the speciation/phylogeographical level in which there is now an abundance of studies, the integration of fossil history and the geological record of physical process tends to be limited, *ad hoc* and/or focused on integration across very recent events, such as the impacts of El Niño or the Pleistocene/Holocene transition. Clearly, broad and powerful studies that extend deeper into the record that integrate broad classes of geological data to address a range of processes responsible for biotic diversification are now possible and should be encouraged.

A second realm of diversity concerns the diversity of morphology as opposed to the diversity of taxa, what Runnegar (1987) termed *disparity*, and the codification of the research programme dedicated to studying disparity has been a subject of contention ever since (Gould 1990, 1993; Briggs *et al.* 1992*a*, *b*; Foote and Gould 1992; Ridley 1993; Foote 1997; Erwin 2007). Progress in understanding the molecular basis of disparity has been less problematic, albeit focused upon individual organ systems. In particular, the developmental genetic underpinnings of the mammalian dentition are so well understood that it has become a model system for understanding organogenesis (Peters and Balling 1999). Fortuitously, the dentition is especially interesting evolutionarily because similarities and differences in dental formulae and morphologies of individual dental positions have been integral to mammalian classification from the beginnings of systematic classification (Owen 1845; Osborn 1907). In addition, the rich fossil record of mammalian dentition can be integrated with phylogenetic schemes of living taxa to underpin its rich evolutionary history (Kielan-Jaworowska *et al.* 2005). Thus, everything is in place to understand why, for instance, the dental classes of incisors, canines, premolars and molars are so stable over geological time, and why there is stability on the composition of dental formulae within clades despite great diversity between living and fossil clades.

The answer to these questions lies with a peculiarity in the way that teeth develop. Mammalian dentition develops through the progressive differentiation of an initially homogeneous dental epithelium (and underlying dental mesenchyme), firstly into the domains of the tooth classes and then the individual tooth rudiments themselves. This is achieved, ultimately, through the interaction of a variety of transcription factors, but repeatedly within the context of a common network of antagonistically interacting cell-signalling molecules (Fgfs and BMPs) (Jernvall *et al.* 2000; Tucker and Sharpe 2004). These are again used in the transition from the differentiated tooth placodes to tooth buds (Neubüser *et al.* 1997), and during tooth morphogenesis, orchestrated via a hierarchy of signalling centres controlling epithelial growth and shape by directing mitotic activity (Jernvall *et al.* 1994, 1998; Vaahtokari *et al.* 1996).

The pattern of progressive differentiation led by reiteration of the same signalling network at multiple levels within the hierarchical pattern of tooth morphogenesis demonstrates how simply diversity can be achieved and also how achieved complexity may be stabilized within an evolutionary framework. Clearly, hierarchical differentiation will inevitably lead to fate restriction, preserving the distinctions between the tooth families in development and evolution. However, anticipated fate can change through shifts in the domains of the transcriptions factors that define tooth class-fate and perturbation of component molecules in the cell-signalling network. For instance, Tucker *et al.* (1998) have effected homeotic transformation of incisor to molar tooth morphology in the mouse through ectopic expression of a BMP signalling antagonist in the presumptive incisor field of the mouse. Thus, *Msx1*, normally positively regulated by BMP4, is not expressed, and *Barx1*, a determinant of molar fate that is normally negatively regulated by BMP4, is unregulated. This experiment echoes natural variation seen within mammals, such as molarized premolars in horses (Butler 1978).

More worryingly for systematists who set any store in the phylogenetic value of dental characters, most experimental manipulations of the component antagonists in the signalling network result in concerted change throughout the dentition, not local effects. This arises directly from reiteration of the same signalling network throughout tooth development. The effects include change in cusp number and position (Kangas *et al.* 2004), tooth number and size, and even variation in dental formulae (Plikus *et al.* 2005), all of which are otherwise so stable and diagnostic of high-level clades (Line 2003). In this light, changed hypotheses concerning the relationships of groups such as Cetacea, previously based heavily on dental characters, should not be surprising (O'Leary *et al.* 2003).

These kinds of studies are not only of general relevance but also have been applied specifically to understanding the developmental basis of, and influence upon, cladogenic events in Deep Time. For instance, targeted analysis of late-stage morphogenesis of teeth in mice and voles has been undertaken specifically to understand the molecular genetic bases of evolutionary patterns recovered from the fossil record, as well their attendant ecological and dietary shifts (Polly 2000). Jernvall and colleagues have demonstrated that these apparently dramatic differences in tooth morphology can result from subtle differences in the timing and position of initiation of signalling centres (Keränen *et al.* 1998; Jernvall *et al.* 2000). The scope for this kind of approach in understanding modest-scale evolutionary change, rooted especially within the context of a populational perspective of development (Jernvall 2000) and a fossil record of morphology (Polly 1998), is potentially limitless (Polly 2000). The dental system has particular advantages for this kind of approach given its excellent fossil record, which tells us so much of the dietary preferences of the animals (Teaford 1988), as well as their ecological and environmental context, revealed not only by their geological context (Renaud *et al.* 2005) but also by the geochemical signatures preserved in their apatite (Grimes *et al.* 2004*a*, *b*).

Finally, simplicity and pleiotropy of this signalling network lends itself to computer modelling. Jernvall and colleagues have developed a simple but realistic morphodynamic model incorporating experimental data to explore tooth morphospace (Jernvall 2000; Salazar-Ciudad and Jernvall 2002, 2005), an approach analogous to Raup's studies of geometrically coiled shells (Raup 1961; Raup and Michelso 1965). Through very subtle variation of the very few parameters incorporated into the model, they were nevertheless able to simulate much of the diversity of mammalian tooth morphology. These analyses must next be extended to the examination of morphospace occupation through time. Once achieved, the results should be more readily interpretable than any previous study of morphospace occupation because of the experimental data on which the limits of realizable morphospace were originally defined, and because dental data are so readily interpretable in functional terms. Clearly, these studies show how integration of developmental genetics and evolutionary morphology can lead to a much more holistic understanding of disparity and how, integrated with geological data with environmental context, this can lead to variance in taxic diversity.

MOLECULAR PALAEOBIOLOGY: A CASE STUDY

The above studies highlight the potential that molecular palaeobiology has for revealing the evolutionary history of life on Earth. For heuristic reasons, we have artificially

divided them into categories like 'genotype' or 'tempo', but the reality is that hard-line distinctions are hard to make for many studies, as they should be for a science coming into its own. Molecular Palaeobiology is an intellectual approach to a set of problems, and we would like to turn now to a set of studies that were not conceived by category, but instead to test hypotheses surrounding the origin of animal phyla, which because it involves the evolution of novel developmental regulatory circuitry in the context of unique environmental circumstances, is ripe for a molecular palaeobiological approach (Conway Morris 1994, 2000*a*; Bromham 2003). Gould (2002), with his usual perspicacity, saw three particularly important issues associated with the Cambrian explosion, and fully advocated a molecular palaeobiological approach to unravelling its Gordian knot of causality: (1) the rapidity of morphological evolution in the Early Cambrian; (2) the cause of this rapidity, whether triggered environmentally or genetically; and (3) the notion of disparity, or the stability of animal body plans over the ensuing 530 myr.

The timing of the radiation

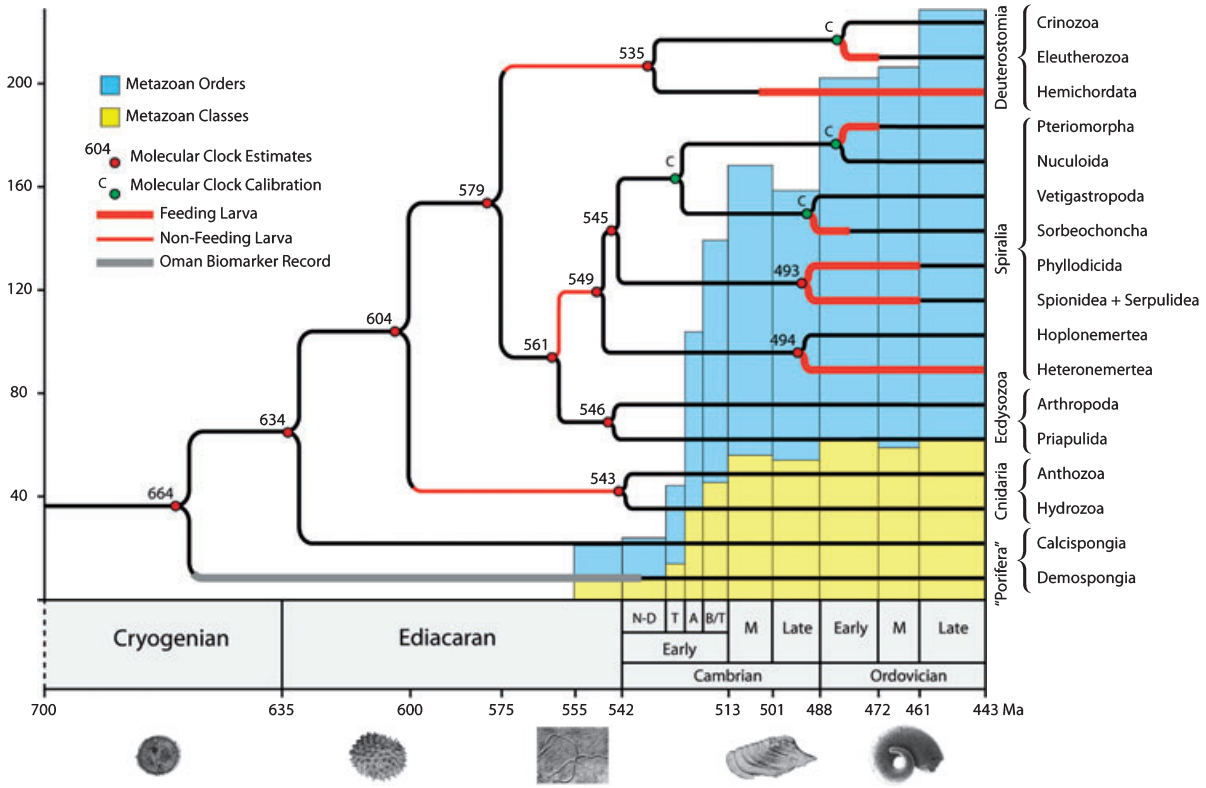
To begin unravelling the causation of the Cambrian explosion, the timing of metazoan radiation must be ascertained (Erwin 1999). Most molecular clock studies, including that of Runnegar (1982), have supported the notion of a deep but cryptic Precambrian history of animals (e.g. Wray *et al.* 1996; reviewed in Conway Morris 2006). The problem with pre-Ediacaran origins for triploblastic metazoans, though, is (at least) two-fold: (1) there is a gap of hundreds of millions of years between supposed taxonomic origination and palaeontological detection (Conway Morris 2006); and (2) these hypothetical animals were ecologically irrelevant until just after the start of the Ediacaran (Peterson and Butterfield 2005). As discussed by these latter authors, the tempo of Precambrian evolution, as assessed by the eukaryotic fossil record, is almost static and does not change until immeditately after the beginning of the Ediacaran (Knoll 1994; Butterfield 2004, 2007). Hence, if these molecular clock estimates are even remotely accurate, then pre-Ediacaran triploblasts not only did not leave any direct record of themselves, they also had absolutely no impact on the surrounding biota, which is most surprising given their unique ability to drive coevolutionary escalation between themselves and other eukaryotes (Butterfield 2007).

Of course, molecular clocks are not without their problems (Rodriguez-Trelles *et al.* 2002; Smith and Peterson 2002; Benton and Ayala 2003; Bromham and Penny 2003; Donoghue and Smith 2003; Graur and Martin 2004; Roger and Hug 2006). Most molecular clock studies deduced the divergence time between the dipteran fly *Drosophila* and the vertebrates using a single calibration point, the divergence between birds and mammals. In addition, most studies used available sequences from public databases; thus, the concordance of estimates could simply reflect that most workers were analysing the same molecules from the same taxa. Peterson *et al.* (2004) and Peterson and Butterfield (2005) took a decidedly different approach: they analysed *c.* 2050 amino acids from seven different housekeeping genes from over 20 metazoan taxa giving multiple calibration points scattered across the metazoan tree (echinoderms, molluscs, insects) and through time (Early Cambrian–Miocene). The use of multiple genes from multiple taxa allowed them to ascertain if the resulting topology was accurate, as assessed by congruence with ribosomal and morphological studies, and to ask if the rate of molecular evolution was similar in three different triploblastic lineages across the Phanerozoic. Not only was their resulting tree accurate (Eernisse and Peterson 2004; Halanych 2004) (Text-fig. 4), they also showed that the rate of molecular evolution in echinoderms, molluscs and insects was similar, and did not change significantly over time. In stark contrast, the rate of molecular evolution in vertebrates was significantly different, accounting for the spuriously deep estimates for the last common ancestor of flies and mice (Peterson *et al.* 2004).

These molecular-clock estimates are in remarkable accord with the known fossil record (Text-fig. 4). The last common ancestor of protostomes and deuterostomes evolved at 580 Ma, and the last common ancestor of metazoans at 665 Ma. In addition, the last common ancestor of cnidarians and bilaterians evolved at 605 Ma, and phylum-level divergences of the three major bilaterian lineages (deuterostomes, spiralians and ecdysozoans), as well as the class-level divergences of Cnidaria, occurred around the Precambrian/Cambrian boundary. Finally, the Porifera are paraphyletic, and the last common ancestor of calcisponges and eumetazoans evolved at 635 Ma.

The trigger

Poriferan paraphyly has important ramifications for both testing the accuracy of the clock and gleaning insight into the cause of the Cambrian explosion itself. Although long considered monophyletic and supported by morphological cladistic analyses (Zrzavý *et al.* 1998; Peterson and Eernisse 2001), molecular phylogenetics have suggested instead that the Porifera are paraphyletic, with calcisponges more closely related to eumetazoans than they are to silicisponges (demosponges and hexactinellids) (reviewed in Sperling and Peterson in press). Sponge paraphyly is particularly important because it suggests that the water-canal system is primitive for both the Metazoa and the



TEXT-FIG. 4. Molecular palaeobiology of the Cambrian explosion. The fossil record of marine invertebrates from the Cambrian through the Ordovician is compared with the divergence estimates of the molecular clock of Peterson and Butterfield (2005). Some of the calibration points are shown ('C') and the divergence estimates are given as determined by Peterson and Butterfield (2005). Also shown is the evolutionary history of feeding larvae as determined by both the molecular clock and the fossil record (see Peterson 2005 and the text for details). Shown at the bottom (from left to right) are the change in acritarch morphology from pre-Marinoan to post-Marinoan (see Peterson and Butterfield 2005), the first appearance of large macroscopic trace fossils (from Budd and Jensen 2000), and the change in morphology of gastropods with non-feeding larvae to gastopods with feeding morphology (from Nützel *et al.* 2006). Data for the metazoan orders and classes are taken from Sepkoski (2002).

Calcispongia + Eumetazoa, and thus these last common ancestors were benthic, sessile, microphagous suspension feeders. Importantly, the design of the water-canal system precludes sponges from feeding on most types of eukaryotes; they feed primarily on bacteria and dissolved organic matter (Brusca and Brusca 2003; Yahel *et al.* 2003, 2005, 2007; Finks *et al.* 2004). Thus, animals living before 634 Ma would have had no ecological impact on the eukaryotic realm, and their existence would be palaeoecologically invisible. Eumetazoans, on the other hand, are characterized by two innovations, a gut and a nervous system. Together these constitute an entirely new grade of organization, macrophagous mobile metazoans, with the potential to have a singularly profound impact on contemporaneous ecology/evolution: for the first time in the history of our planet, large eukaryotes could now prey on other eukaryotes (Peterson and Butterfield 2005).

The molecular clock estimates that this new grade of organization arose sometime between 634 and 604 Ma (Peterson and Butterfield 2005). This estimate is coincident with a major regime change in the Proterozoic acritarch record, including: (1) the disappearance of low-diversity, evolutionarily static, pre-Ediacaran acritarchs; (2) a radiation of the high-diversity, short-lived Doushantuo-Pertatataka microbiota; and (3) an order-of-magnitude increase in evolutionary turnover rate. This then constitutes a true revolution in the fossil record: the end of evolutionary stasis, and the beginning of both rapid evolutionary turnover and rapid morphologically complex coevolution (Knoll 1994; Butterfield 2004, 2007; Peterson and Butterfield 2005).

Peterson and Butterfield (2005) argued that the macroevolutionary regime change that occurs after the Marinoan, and represents the beginning of what will become the Phanerozoic, is a direct consequence of the evolution of eumetazoans, in particular the unique ecological challenges and coevolutionary consequences accompanying the acquisition of a gut. They further argued that these clock estimates were accurate because the more readily preserved microfossil record provides positive evidence

for the absence of pre-Ediacaran eumetazoans, and the presence of eumetazoans soon after the beginning of the Ediacaran. Nonetheless, the accuracy of this molecular clock, which has been questioned by some (e.g. Blair and Hedges 2005), can be assessed independent of these ecological arguments, by returning to the direct fossil record of animals themselves. It has long been known that there is a distinctive steroid biomarker pattern in Neoproterozoic–Cambrian sediments and petroleum (McCaffrey *et al.* 1994; Love *et al.* 2006) suggestive of input from demosponges. Two molecules in particular, 27-norcholesterol and 24-isopropylycholesterol, and related sterols with the same C_{26} and C_{30} skeletons, are present in modern demosponges from a wide range of clades, but are not biosynthesized by Bacteria or Archaea (including those with apparent symbiotic association with, or located within the sponges themselves), or from calcisponges (Love *et al.* 2006). Love *et al.* (2006) have now shown in the South Oman salt basin, where organic-rich sediments range in age from >635 to <542 Ma, that there is a continuous and unambiguous record of elevated abundances of both demosponge markers. This work is more rigorously constrained than an earlier report (McCaffrey *et al.* 1994), especially with respect to the ages of the samples, their palaeoenvironmental settings and the techniques used to attribute the biomarkers to the host rocks. With respect to the latter, Love *et al.* (2006) showed that the sponge-diagnostic steroid hydrocarbons could be generated directly from *in situ*, macromolecular organic matter (kerogen) and therefore cannot have migrated from younger or older rocks (Love *et al.* 1995). They found no evidence for any kerogen-bound sponge biomarkers in any pre-Sturtian rocks, suggesting that crown-group Demospongia arose after the Sturtian, minimally *c.* 657 Ma (Kendall *et al.* 2006). This is in remarkable accord with the molecular clock that dates the origin of these biomarkers to sometime between 664 and 632 Ma (Peterson and Butterfield 2005; see Text-fig. 4). Therefore, two independent data sets, the palaeoecology of eukaryotes and the biomarker record of demosponges, suggest that the divergence times shown in Text-figure 4 are robust.

These data suggest that it was the origin of predators that was the ultimate trigger for the Cambrian explosion, consistent with many other workers' insights and arguments (e.g. Stanley 1973; Bengtson 1994, 2004; Butterfield 1997, 2001). The paraphyly of poriferans strongly suggests that the origin of both the gut and the nervous system occurred within the context of sponge biology and evolution, and was in place sometime between 634 and 604 Ma (Text-fig. 4). The fact that the origin of the eumetazoan ground plan occurred on the heels of the Marinoan Snowball Earth episode may not be a coincidence: such an environmental upheaval would surely have had some impact on the life histories of some subset of the biota. The fact that several supposed eumetazoan apomorphies, including true tissues, are present in the larval stages of some sponges (reviewed by Sperling and Peterson in press) suggests that neoteny might underlie the evolution of the eumetazoan grade of organization from a sponge-like larva (Maldonado 2004). Thus, the extreme oligotrophic conditions present during and immediately following the Marinoan (Hoffman *et al.* 1998) may have exerted a profound selection pressure upon sponges to increase the time spent searching for suitable substrate, and thus for larval feeding. Indeed, the only modern clade of sponges to feed directly upon other animals, the cladorhizids, which have lost the water-canal system and feed macrophagously upon mesozooplankton using a derived mode of extracellular digestion, live in extreme oligotrophic environments (Vacelet and Boury-Esnault 1995). The concordance between the observations that cladorhizids live in oligotrophic environments, and that eumetazoans arose on the heels of the Marinoan glaciation, may be of some significance (Vacelet and Duport 2004).

The impact of predation on metazoan evolution and the evolution of complex life cycles

One important consequence of introducing benthic, mobile, macrophagous predators into the world's ecology might have been the evolution of zooplankton, as small animals, as well as the embryonic stages of larger animals, exploited the initially predator-free pelagos for safety (Signor and Vermeij 1994; Butterfield 1997, 2001; Rigby and Milsom 2000; Peterson *et al.* 2005). As detailed by Butterfield (1997, 2001), the introduction of small animals into the water column changed the pelagos from a relatively simple to a much more complex ecosystem and, by linking this newly established pelagic ecosystem with the benthos, effectively established the 'modern' or Phanerozoic ocean. Butterfield, following upon the ideas of Stanley (1973), further argued that it was the invention of marine ecology itself that was the cause of the Cambrian explosion.

Because the molecular clock is accurate, we can use it to date when various types of mesozooplankton, specifically primary marine larval forms, evolved. Peterson (2005) argued that three different larval forms, the dipleurula of ambulacrarian deuterostomes (i.e. echinoderms and hemichordates), the trochophore of spiralians and the planula larva of cnidarians, arose sometime between the latest Precambrian and the Early Cambrian, coevally with the first appearance of macroscopic trace fossils (Text-fig. 4). Hence, the estimates derived from the molecular clock are consistent with the prediction (Signor and Vermeij 1994; Butterfield 1997) that pelago-benthic

life cycles are of great antiquity, evolving at least three times independently during the late Ediacaran–Early Cambrian, possibly as a result of the rise of benthic predation (Text-fig. 4).

Consistent with the fossil record of molluscs (Nützel *et al.* 2006; Text-fig. 4) and data derived from comparative gene-regulatory-network analysis (Dunn *et al.* 2007), the molecular clock, coupled to the metazoan tree, suggests that the evolution of feeding larvae, or planktotrophy, occurred in multiple clades starting in the latest Cambrian and was probably achieved by the mid-Ordovician (Peterson 2005). This convergent evolution of planktotrophy was probably a response to the advent of tiered suspension feeders such as pelmatozoan echinoderms and rugose corals, which themselves evolved in response to this newly plentiful food source in the plankton (Signor and Vermeij 1994; Peterson 2005). Hence, mesozooplankton evolved in response to benthic predators, which in turned provided a food source for not only macrozooplankton and nekton (Vannier *et al.* 2007) but also epifaunal suspension feeders, which in turn exerted a selection pressure for the evolution of fecundity and thus of planktotrophy (Peterson 2005; Peterson *et al.* 2005).

This macroevolutionary interplay between planktonic larvae and benthic predators may go some way to explaining the dramatic Ordovician biodiversification event. Besides the coupled changes to the taxonomic compositions of the pelagos and benthos, the time larvae spend in the plankton feeding has the effect of increasing the taxon's geographical range and thus increasing the amount of gene flow, as compared with closely related lecithotrophs (McMillan *et al.* 1992; Hellberg 1996; Collin 2001). The evolution of lecithotrophy, then, has the effect of facilitating the isolation of populations, possibly leading to the formation of new species (Shuto 1974). Whether the macroevolutionary implications of this, assuming there are any (Jeffery *et al.* 2003), are the result of species selection (Hansen 1978, 1982; Arnold and Fristrup 1982; Jablonski and Lutz 1983; Jablonski 1986), species sorting (Duda and Palumbi 1999; Lieberman *et al.* 1993; Hart 2000) or possibly some combination thereof (Jeffery and Emlet 2003) remains unclear. Nonetheless, the exploitation of the pelagos may explain, in part, why taxa exhibited significantly greater longevity and larger geographical ranges during the Late Ordovician as compared with the Early Ordovician (Miller 1997, 2004).

The evolution of metazoan morphological complexity

The data summarized above go some way toward addressing the first two issues, that of the timing and the trigger, but the one issue not explored by these studies is the third leg of the Gouldian triumvirate: the origin and subsequent stability of animal body plans over geological time. Body plan stability is, according to some (Levinton 2001), the single most important fact the fossil record has contributed to the science of evolutionary biology. Unlike plants, which continue to evolve new grades of construction over time, resulting in the paraphyly of most major plant groups (Donoghue, M. J. 2005), all major animal phyla appeared early in the evolutionary history of metazoans (Erwin *et al.* 1987) and aside from sponges (see above), rotifers (Welch 2001; Garcia-Varela and Nadler 2006; Sorensen and Giribet 2006) and possibly annelids (Halanych *et al.* 2002; Bleidorn *et al.* 2003*a*, *b*; Jordens *et al.* 2004), all phyla are monophyletic.

One way to address the evolutionary dichotomy between plants and animals is to characterize the genetic toolkit responsible for building the body plans of each. One of the major surprises from comparative genomic studies is that during animal evolution genomic complexity long preceded the advent of morphological complexity (Technau *et al.* 2005). Morphologically complex animals like protostomes and deuterostomes are characterized by the possession of organs and organ systems, structures that are absent in more basal taxa including cnidarians and sponges. However, comparative genomic studies have revealed that the metazoan genome is fairly stable, especially with regard to the developmental toolkit, since most animals have in the neighbourhood of 20,000 protein-coding genes. Not only is the developmental repertoire much the same between protostomes and deuterostomes (Materna *et al.* 2006) but also much of this complexity extends into cnidarians (Kortschak *et al.* 2003; Kusserow *et al.* 2005; Magie *et al.* 2005; Technau *et al.* 2005; Kamm *et al.* 2006; Materna *et al.* 2006; Matus *et al.* 2006; Ryan *et al.* 2006, 2007; Simionato *et al.* 2007), and even sponges (Jager *et al.* 2006; Larroux *et al.* 2006; Nichols *et al.* 2006; Simionato *et al.* 2007). It appears then that a fairly complex set of transcription factors and signalling networks is necessary for the evolution of multicellularity. Indeed, in the sea urchin *c.* 80 per cent of the transcription factors (excluding the zinc-finger genes) (Howard-Ashby *et al.* 2006) and *c.* 50 per cent of the entire protein coding capacity (Samanta *et al.* 2006) is used within the first 48 h of development to build a relatively simple embryo (see also Wei *et al.* 2006). Thus, the remarkable jump in morphological complexity associated with protostomes and deuterostomes is divorced from any jump in mRNA complexity, recently christened the g-value paradox by Hahn and Wray (2002; see also Taft *et al.* 2007). In addition, within protostomes and deuterostomes, morphological disparity was achieved using this same basic toolkit. So how was morphological complexity achieved, and how was morphological disparity generated in such a rapid manner (a few tens of millions of years at best) without any obvious changes to the repertoire of

transcription factors and other developmentally relevant classes of genes?

Of course, part of the answer must lie with how the toolkit is wired together in each group of animals (Carroll *et al.* 2001; Davidson 2001; Davidson and Erwin 2006). Nonetheless, it might be worth turning our attention to other sorts of regulatory genes beyond the mRNA repertoire. One new group of regulatory genes that might be part of the solution to the Cambrian explosion conundrum are miRNAs, the *c.* 22 nucleotide regulatory RNA molecules discussed above. miRNAs regulate a tremendous number and diversity of biological processes, and one of the more interesting roles they play during animal development is controlling the fidelity of gene expression and maintenance of cell-lineage identity during histo- and organogenesis (Brennecke *et al.* 2005; Chen and Meister 2005; Giraldez *et al.* 2005; Stark *et al.* 2005; Zhao *et al.* 2005, 2007). miRNAs accomplish this by regulating combinatorially tens to hundreds of target genes per each miRNA (reviewed in Bentwich 2005; Rajewsky 2006). In other words, rather than controlling the patterning of body plans *per se*, it is thought that miRNAs act rather as 'lock-down' mechanisms on cellular differentiation (Chen and Rajewsky 2007).

Despite their critical role in gene regulation, aside from the phylogenetic conservation of a handful of miRNAs in flies, nematode worms and vertebrates, little is known about the evolution of miRNAs themselves. Nonetheless, a critical observation was made by Pasquinelli *et al.* (2000, 2003): the only metazoan miRNA studied thus far from a broad phylogenetic perspective, *let-7*, was detected in many different protostomes and deuterostomes, but not in any basal group of animals nor in any non-metazoan genome, and it has not been secondarily lost in any system examined to date. Sempere *et al.* (2006) confirmed this phenomenon for many of the miRNAs in the fly and human. Of the 20 miRNAs that characterize protostomes and deuterostomes, only two are present in cnidarians, and an additional five are present in acoel flatworms (Text-fig. 2). What is compelling is that miRNAs associated with specific organs (e.g. miR-1: heart; miR-9: brain) appear not to be present in cnidarians or acoel flatworms: organ-specific miRNAs are only in taxa that have organs. In addition, no miRNAs were found in sponges (Sempere *et al.* 2006; see also Prochnik *et al.* 2007) or ctenophores (KJP, unpublished data), suggesting that the evolution of miRNAs occurred at the base of Bilateria (Cnidaria + Triploblastica; see Peterson *et al.* 2005). Sempere *et al.* (2006; see also Hertel *et al.* 2006) further showed that miRNAs were continuously added to metazoan genomes through geological time: protostomes have at least 12 additional miRNAs, deuterostomes seven, arthropods two, chordates four, and Osteichthyes *c.* 75 miRNAs (Text-fig. 2). Hence, complex animals have miRNAs not found in simpler animals, and unique body plans possess unique miRNAs. Complexity, then, as discussed by Taft *et al.* (2006), may entail the expansion of both *cis*-acting regulatory elements (Davidson and Erwin 2006) and *trans*-acting non-coding RNAs (Sempere *et al.* 2006; Niwa and Slack 2007) integrated in a precise and elegant way so that novel phenotypes arise without disrupting the existing regulatory networks (Chen and Rajewsky 2007).

Thus, these studies have shown the following:

Genotype. Poriferans are paraphyletic, leading to the conclusion that the last common ancestor of metazoans was a sponge, and that the origin of eumetazoans, and hence the origin of both the nervous system and the gut, occurred in the context of sponge biology and evolution.

Phenotype. The evolution of new cell types, and ultimately the origin and stability of body plans, might be governed, to some degree, by the evolution of new miRNAs.

Environment. Eumetazoans arose on the heel of Snowball Earth, suggesting that the extreme oligotrophic conditions that might have prevailed during the Marinoan glaciation interval may have selected for one group of sponges to be able to feed using an archenteron.

Tempo. A molecular clock can be used to infer the divergence times of metazoan taxa, and thus the rate of genic evolution can be strongly disassociated from the rate of morphological evolution.

Mode. Larval-governed macroevolution is ultimately a product of predator-prey interactions that arose in the Early Ordovician, resulting in the evolution of planktotrophic taxa.

Diversity. The Cambrian explosion is a real biotic event and not an artefact of an incomplete fossil record.

A BRIEF APOLOGIA FOR OUR EMPHASIS ON ANIMAL EVOLUTION

Our myopic focus on animal evolution in this review reflects, in part, our knowledge base rather than the opportunities for molecular biology in plant evolution. However, it is also a reflection of the fact that plant evolutionary developmental biology has lagged considerably (Friedman *et al.* 2004). An immense amount of work has been undertaken on the model system *Arabidopsis*, a eudicot angiosperm, to understand the molecular basis of root, shoot, leaf and flower specification and growth.

However, a general understanding of the origin and evolution of angiosperm development is hampered by the failure to resolve their nearest living relatives among the seed plants (Friedman and Williams 2004; Palmer *et al.* 2004; Bateman *et al.* 2006; Doyle 2006; Hilton and Bateman 2006). Thus, attempts to infer conserved developmental bases of organs are obscured by false and unrecognized homologies, all for the lack of a suitable out-group. Resolving the origin of angiosperms is as abominable today as it was for Darwin.

Nevertheless, the basis of a candidate gene approach is in place and surveys of transcription factors implicated in flower and leaf development, such as members of the homeobox and MADS-box families of transcription factors, have already been undertaken in the successive sister clades of angiosperms, the gymnosperms (Becker *et al.* 2003), ferns (Munster *et al.* 2002), mosses (Krogan and Ashton 2000; Champagne and Ashton 2001; Henschel *et al.* 2002; Zobell *et al.* 2005) and even charophycean green algae (Tanabe *et al.* 2005). These surveys have revealed that the diversification of embryophytes has been accompanied by multiple rounds of gene duplication to account for the entire clades of homeobox and MADS-box genes encountered in crown-angiosperms (Munster *et al.* 2002; De Bodt *et al.* 2005). These data have also been employed in attempts to understand the evolution of the regulatory network underpinning angiosperm flower development (Theissen *et al.* 2002) and in speculative attempts to explain the origin of angiosperms from their gymnosperm relatives (Becker *et al.* 2003).

The study of plant evolution is well placed to unleash a molecular palaeobiological revolution. Not only is it technically feasible to extend knowledge of angiosperm developmental genetics to the remaining embryophytes but, in contrast to the controversial and almost non-existent (or unrecognized) record of stem-angiosperms (Sun *et al.* 1998, 2002; Friis *et al.* 2003), there is a rich record of stem-tracheophytes, as well as lycopsids, ferns, horsetails and gymnosperms that is well understood in terms of character evolution (Kenrick and Crane 1997; Donoghue, M. J. 2005). Furthermore, there is a wide variety of hypotheses of homology, as well as explanations of proximal and ultimate causation for the origin of these characters that are available for testing (Friedman *et al.* 2004). This is especially true of gross plant architecture, the universe of which has been mapped and over which random walks have traversed to identify the relative fitness of this landscape (Niklas 1982, 1994, 1999). These studies have also been employed to test for, and reject the role of, for instance, developmental constraints, in restraining lineages from attaining well-adapted morphologies (Niklas 1997).

The main limitation to opening up this area of research is the problem of plant phylogeny, which remains unresolved at a very gross level (Palmer *et al.* 2004). This is especially true of the relationships of bryophytes to each other (Shaw and Renzaglia 2004), as well as the interrelationships of seed plants (Bateman *et al.* 2006; Doyle 2006; Hilton and Bateman 2006) and, most disconcertingly, the closest living relatives of angiosperms among the seed plants (Friedman and Williams 2004; Palmer *et al.* 2004; Bateman *et al.* 2006). This impacts also upon attempts to infer the timing of diversification of the major plant groups, which remain polarized between deep molecular clock and shallow palaeontological estimates (Wellman 2003) and, thence, determining the environmental context and consequences of these events. Thus, the molecular palaeobiology of plants is in much the same state as metazoan evolutionary history was a decade or so ago. However, this decade of research into metazoan molecular palaeobiology provides a model for an integrative elucidation of plant evolution.

MAKING MOLECULAR PALAEOBIOLOGY HAPPEN: WHAT DOES IT ENTAIL?

The examples given above bring together disparate, but by no means exhaustive, sources of data, and our aim has been to show that these can be integrated into a holistic view of organismal evolutionary history. Indeed, the examples and case study demonstrate that this approach is already being taken by a number of scientists, providing corroborative support for the component strands of their research and, in combination, creating entirely new hypotheses and perspectives on evolutionary history. Some studies, such as Jukka Jernvall's analyses of dental ontogeny and phylogeny, rooted in the context of functional, ecological and environmental change, even suggest that a rapprochement may be achievable between the objectives of evolutionary developmental biology and the neo-Darwinian synthesis, rooted in population genetics (Amundson 2005). What is more, palaeontological data will be integral to this endeavour.

Although most of the examples discussed above demonstrate how molecular data have shone new light on thorny palaeontological problems, it is not our view that the relationship between palaeontological and molecular data is so polarized. Rather than a symptom of molecular chauvinism, our examples serve to demonstrate the relevance of molecular data to palaeontology and the novelty of these molecular data vs. the almost timeless patterns that have emerged from palaeontological data has, for the moment, generated a rather lopsided approach. Indeed, molecular biology has only addressed macroevolutionary questions comparatively recently, and can claim for its own relatively few macroevolutionary hypotheses testable

by the addition of palaeontological data. Nonetheless, this situation is changing (see below), and molecular biologists are increasingly aware of the pivotal role that palaeontological data can play in unravelling molecular, developmental and, indeed, organismal evolution.

Palaeontologists hit the field

The potential for this kind of integrative science is great and does not necessarily require that its proponents don lab coats and own a fist-full of micropipettes. Although the explicit goal of molecular palaeobiology is to integrate the molecular approaches to address questions that have traditionally been within the remit of palaeontology, palaeontological data and their geological context will always be fundamental because of the unique role that they play in constraining our understanding of organismal and therefore molecular and developmental evolution. This was foreseen as early as 1981 by Colin Patterson when he considered the importance of palaeontological data in systematics, and outlined the unique functions they played: (1) provision of a timescale of minimum ages of groups; (2) amplification of information on distribution in space; (3) prediction of the sequence in which crown-group homologies arose; and (4) stem-group fossils added to the system of relationships providing valuable tests of the recent groupings by revealing unsuspected characters or combinations of characters that may necessitate reversals of decisions on polarity and homology.

These points were amplified and extended by Andrew Smith (1998) who demonstrated that the impact of increased taxonomic sampling provided by addition of fossil taxa to phylogenies of the living: (5) improved the accuracy of tree reconstruction through the recognition of otherwise cryptic homoplasies; (6) resolved branching order where internodes are short and branches are otherwise long; and (7) resolved the origin of highly derived characters.

Many of these points centre on the multifarious roles that fossils play when arrayed systematically among their living relatives. These can be summed, in the main, within what is recognized as the stem-group (Jefferies 1979; Hennig 1981), originally erected as a taxonomic dustbin for fossil taxa that failed to exhibit the full inventory of diagnostic characters necessary for inclusion within the concept of a clade defined on the basis of its living members, which is in turn known as the crown-group (Jefferies 1979). Thanks especially to the work of Dick Jefferies, the concept of the stem-group has been transformed to nested sets of crown-group sister-clades, revealing the sequence of assembly of crown group characters (Budd 2001*a*; Donoghue, P. C. J. 2005). Thus, stem-groups have taken on the role of a Rosetta Stone for uncovering cryptic homology and therefore character transformations, as well as the relative order of acquisition of evolutionary novelties between living sister-groups separated by long branches. By augmenting comparative anatomical data from living intermediates of molecular models, such patterns are integral to constraining explanatory models of developmental evolution (Budd 2001*a*, *b*; Donoghue, P. C. J. 2005).

Palaeontological data organized in this manner can provide dramatic tests of established hypotheses of developmental and molecular evolution. Explicit phylogenetic recapitulation was long ago rejected (Garstang 1928), but it remains a potent influence on developmental models for the origin of novel characters. Hence Tabin argued that the paired fins of vertebrates evolved first in a pelvic position, only subsequently appearing in the pectoral position after subdivision of a rostral extension of its anlagen in development and evolution (Tabin 1992; Tabin and Laufer 1993). Stem-gnathostomes demonstrate that the pattern of acquisition is identically opposite (Coates 1993, 1994). At an even broader level, the condensation of characters uniting extant clades, resulting from the extinction-driven creation of their stems, has led to hypotheses that thresholds of complexity were crossed at the origin of crown vertebrates, crown gnathostomes and crown teleosts (Holland *et al.* 1994; Sidow 1996; Aburomia *et al.* 2003). These crown-clades also coincide with events of widespread gene or genome duplication and inevitably a hypothesis of causality has emerged from this coincidence of genotypic and phenotypic phenomena (Panopoulou and Poustka 2005; Volff 2005; Brunet *et al.* 2006). Of course, once stem-taxa are brought into consideration, the phenomenon of increasing complexity is seen to be an artefact of extinction or, at the very most, the product of a protracted episode of gradual character acquisition that is certainly not compatible with the genome duplication-driven hypothesis of causality as originally formulated (Donoghue and Purnell 2005).

Reconstructed stems can help in resolving much more fundamental patterns of body plan evolution, the most dramatic of which is the origin of pentameral echinoderms from bilateral ancestors (Mooi and David 1998). Evidence from molecular developmental genetics may reveal what is possible concerning the adaptation of existing patterning mechanisms, but only the stem can reveal what actually happened, constraining alternative models of developmental evolution (Peterson *et al.* 2000). It has been widely accepted that at least some stem-echinoderms deviated from pentamery and that some forms, such as the helicoplacoids, exhibited triradial symmetry (Paul and Smith 1984). The early history of symmetry deviancy in echinoderm lineages has not been clear because of controversy surrounding the interpretation of the carpoids (Jefferies 1986, 1997; Peterson 1995; Ruta 1999; David

et al. 2000). Smith (2005) has recently reinterpreted the comparative anatomy and phylogenetic relations of these echinoderm-like fossils in light of the discovery that echinoderms and hemichordates are each others' closest relatives (Smith *et al.* 2004), and the discovery that the 'stem' of the carpoid is not a feeding arm but a muscular locomotory organ (Clausen and Smith 2005). His conclusion is that the carpoids are a paraphyletic grade of stem-echinoderms that retain many symplesiomorphies of crown-deuterostomes, hitherto perceived as chordate (or hemichordate + chordate) characters before the recognition of Ambulacraria. Together with cinctans, they provide an anatomical framework for understanding the establishment of the echinoderm bodyplan using molecular developmental genetics.

The systematic organization of fossil taxa into stems and crowns can also be used to understand the origin of character complexes. In particular, Budd has used his framework of stem arthropods to understand the origin of segmentation in functional terms (Budd 1998, 2001*c*). He has provided a compelling argument for the gradual assembly of the 'segment' and of arthropodization that runs contrary to the atomistic perspective of segmentation evolution that pervades evolutionary developmental biology. Thus, despite the rather slow application to organizing extinct invertebrates (Donoghue, P. C. J. 2005), the stem-group and its allied suite of concepts are incredibly powerful vehicles for elucidating the phylogenetic significance of fossil taxa with respect to their living relatives, and as such demonstrate the integral role that palaeontological data and theory play in molecular palaeobiology.

Palaeontologists hit the lab

While it is true that it is not necessary to pick up a pipette to join the party, the exploitation of molecular data and techniques by palaeontologists offers an approach to addressing palaeontological problems that remains largely unexploited. The key question in achieving this melding of palaeontological data and problems with molecular data and techniques is whether molecular biologists should be brought into palaeontology, or whether palaeontologists should develop laboratory skills and an appreciation for molecular data.

This is not a new question (Roush and Pennisi 1997) and the answers that have been provided by the palaeontological and molecular communities have been predictably partisan. There are clear advantages to drawing molecular biologists into molecular palaeobiology: they have the necessary laboratory skills and ability to determine which questions are tractable within the universe of existing techniques and the limitations inherent in some organisms. One drawback is that the majority of molecular biologists are trained to work on model laboratory organisms such as mouse, chicken, zebrafish or *Drosophila*. However, model systems were established for reasons other than their evolutionary significance (Bolker and Raff 1996), and the extension of established skills to non-model systems can be non-trivial. Indeed, many of the approaches employed in model systems, such as the establishment and analysis of transgenic lines, are unlikely to be appropriate for molecular palaeobiology. Furthermore, this kind of training does not engender an appreciation of phylogenetics, biodiversity, geological time, the nature of the fossil record or, indeed, of fossil data (Runnegar 1986). On the other hand, palaeontological training rarely leads to experience of molecular data: how to obtain, manipulate and interpret them. However, the challenges to members of both communities are far from being insurmountable. The key driver is a desire to answer the kinds of questions that we have outlined as falling within the sphere of Molecular Palaeobiology. There are already a number of laboratories around the world, based in biology and geology departments, where laboratory-based research of the kind that we call molecular palaeobiology is practised, employing scientists from backgrounds in the earth sciences, comparative anatomy, classical embryology and palaeontology.

Thus, there are few pragmatic limitations to palaeontologists obtaining practical skills in molecular biology, and with these in hand they may approach some of the classical questions on palaeontological science anew. This would include attempts to understand the mechanistic basis, in terms of the evolution of regulatory interactions and expression domains, of the patterns recovered from the organization of fossil taxa into stem-groups, thereby explaining the origin of their respective crown groups from both pattern and process perspectives, e.g. the origin of echinoderm pentaradial symmetry (Peterson *et al.* 2000), assembly of the arthropod head organization (Budd 2002; Eriksson *et al.* 2003), the origin of the molluscan skeleton (Jacobs *et al.* 2000), the origin of vertebrate fins (Freitas *et al.* 2006) and limbs (Metscher *et al.* 2005) or, indeed, the timing of such innovations (Peterson *et al.* 2004). Finally, integrated with geological data, such temporally constrained data on evolutionary patterns and mechanisms may be interpreted in light of their ecological and environmental context, providing a more holistic, molecular palaeobiological, perspective (Peterson and Butterfield 2005).

CONCLUSIONS

Twenty-one years have elapsed since Bruce Runnegar published his codification of Molecular Palaeontology and so this discipline should now have come of age. Palaeontological data are routinely marshalled in molecular

analyses of the origin of evolutionary novelties, and of evolutionary rate, but this kind of integrative research has been stimulated primarily by molecular and developmental biologists addressing evolutionary questions that have traditionally been considered palaeontological. More than ever before, palaeontologists and molecular biologists interact, but these groups of scientists remain largely immiscible, polarized along partisan lines in debate.

As we have argued throughout, the geological and genetic records of evolutionary history are directly compatible and complementary, and there can be little doubt that molecular data have breathed life into historical debates that would otherwise have remained mired in conflict between embryological, histological, anatomical and palaeontological data (Scotland *et al.* 2003). Nevertheless, no single data set has a preserve over the truth in attempts to uncover evolutionary history (Jenner 2004) and, indeed, if our objective is to understand organismal, rather than merely molecular, evolution we have no recourse other than to take a fully integrative approach.

The challenge to palaeontology (and to palaeontologists) is to recapture its traditional domain of scientific enquiry by embracing molecular data, techniques and the molecular approach. We have set out examples of how this kind of integrative approach is already underway, and we provide a synthetic framework for a new, Molecular Palaeobiology. This requires palaeontologists to think in much the same way that they have always done, but now to obtain the technical skills of molecular biologists. The potential rewards are great: competence in all of the relevant data, techniques and approaches necessary to reveal the full tapestry of life on Earth. However, molecular palaeobiology is an opportunity that cannot afford to wait for another generation.

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