

Coral development: from classical embryology to molecular control

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ABSTRACT The phylum Cnidaria is the closest outgroup to the triploblastic metazoans and as such offers unique insights into evolutionary questions at several levels. In the post-genomic era, a knowledge of the gene complement of representative cnidarians will be important for understanding the relationship between the expansion of gene families and the evolution of morphological complexity among more highly evolved metazoans. Studies of cnidarian development and its molecular control will provide information about the origins of the major bilaterian body axes, the origin of the third tissue layer, the mesoderm, and the evolution of nervous system patterning. We are studying the cnidarian *Acropora millepora*, a reef building scleractinian coral, and a member of the basal cnidarian class, the Anthozoa. We review our work on descriptive embryology and studies of selected transcription factor gene families, where our knowledge from *Acropora* is particularly advanced relative to other cnidarians. We also describe a recent preliminary whole genome initiative, a coral EST database.

KEY WORDS: *Acropora*, *Pax*, nuclear receptor, *Hox*, *EST*

Introduction

The rationale for using a cnidarian to study gene evolution is clear from the idealised metazoan phylogenetic tree shown in Fig. 1. The Cnidaria form an outgroup to the Bilateria, the two groups having diverged from a common ancestor, probably sometime between 500 and 600 million years ago (Valentine *et al.*, 1999). There is, of course, no way to directly assess the gene complement of this common ancestor, or to determine which gene regulatory networks and developmental pathways it possessed. However, comparisons of the genes, networks and pathways of extant cnidarians and higher metazoans can provide considerable information if it is assumed that shared characters were present in the common ancestor. This rationale forms the basis of the work described below.

As a model primitive metazoan, the reef building coral, *Acropora millepora*, offers a number of advantages over the more widely studied freshwater cnidarians, *Hydra* and *Chlorohydra* (Miller and Ball, 2000), not least of which is the availability of embryonic material. *Hydra* commonly reproduces by asexual budding while sexual reproduction is unpredictable and much of embryonic development occurs within a thick cuticle, making study difficult

(Martin *et al.*, 1997). In contrast, large quantities of relatively synchronous embryos can be collected from annually spawning reef corals such as *Acropora* (Harrison *et al.*, 1984, Babcock *et al.*, 1986). This is a vital resource for laboratories such as ours, facilitating studies on a number of aspects of coral biology. First, and most fundamentally, a major thrust of our research is to investigate the molecular mechanisms of coral development from an evolutionary point of view (pure "evo-devo" (evolution of developmental mechanisms) *sensu* Raff, 2000). Other major interests are to investigate at cellular and molecular levels the profound reorganisation of the body that occurs when the coral planula larva settles to form a polyp, and the processes of calcification and uptake of symbiotic photosynthetic microalgae. The stress-induced release of these symbionts was responsible for the much publicised worldwide coral bleaching episodes in 1997-1998 and previously (e.g. Wilkinson, 1998). Apart from their intrinsic interest, these latter studies have important ecological and economic implications.

Abbreviations used in this paper: EST, expressed sequence tag; SEM, scanning electron microscope.

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Due to the relatively recent discovery of coral mass spawning (Harrison *et al.*, 1984), the extensive embryological and morphological information available for many species does not yet exist for corals, so descriptive embryology, anatomy, and molecular biology are all proceeding in parallel. In the following pages we first summarise what is known about morphological development and differentiation during coral embryogenesis. We then review our recent work on cnidarian Pax genes, nuclear receptor genes and Hox-like genes. For the former two groups of genes more information is available for *Acropora* than for any other cnidarian. We end with a discussion of an expressed sequence tag (EST) project which, when expanded to include microarrays, should speed our understanding of the roles and interactions of various genes in *Acropora* embryonic development.

Descriptive Embryology

Accounts of the morphological development of several coral species are now available. Early work was summarised by Harrison and Wallace (1990), while more recent work, specifically on *Acropora* species, was presented by Hayashibara *et al.* (1997). Figure 2 shows drawings of a series of the major morphological stages of embryonic development of *Acropora millepora* (*A. millepora*), together with the approximate time required to reach those stages under conditions prevailing near Townsville, Queensland in 1997. The speed of development is temperature dependent, and temperature varies from year to year, but these times are within the typical range. There are several developmental stages that are worthy of particular comment. The first cleavage division is equal and holoblastic. Cleavage occurs by progressive furrow formation; the cleavage furrow initiates on one side of the fertilised egg, and moves across to the opposite side resulting in the formation of two equal blastomeres. In hydroids, which also

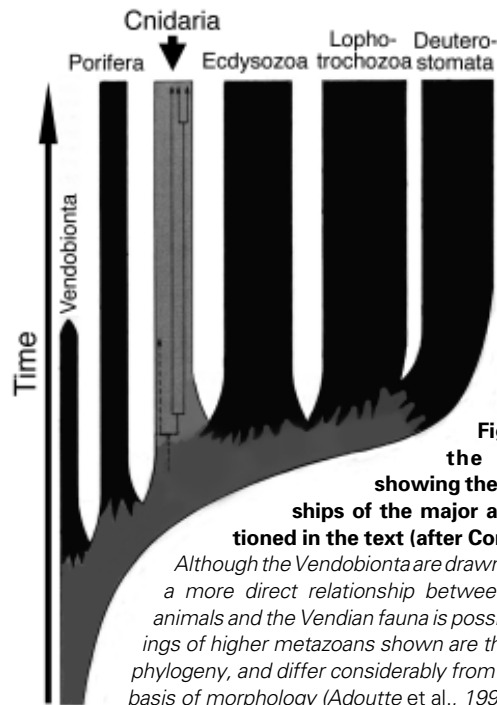


Fig. 1. A phylogeny of the animal kingdom showing the presumed relationships of the major animal groups mentioned in the text (after Conway Morris, 2000).

Although the Vendobionta are drawn as an extinct lineage, a more direct relationship between extant diploblastic animals and the Vendian fauna is possible. The major groupings of higher metazoans shown are the result of molecular phylogeny, and differ considerably from those created on the basis of morphology (Adoutte *et al.*, 1999).

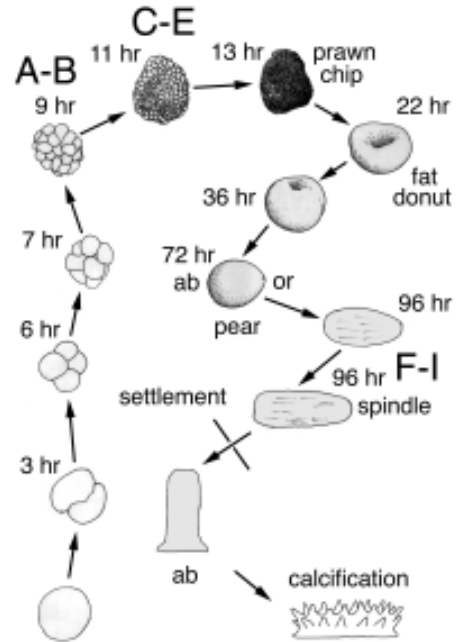


Fig. 2. Diagrammatic summary of the major morphological stages in the embryonic development of *A. millepora*. Development times shown are typical for this species on the northern Great Barrier Reef of Australia, although there may be considerable variation from year to year, depending on the temperature. The letters relate to micrographs of comparable stages shown in Fig. 3. Modified from Ball *et al.* (2002).

show a progressive furrow formation during the first cleavage, the site of furrow initiation corresponds to the future posterior pole of the larva, indicating that specification of the body axis has already occurred (reviewed in Goldstein and Freeman, 1997). The similarity between the first cleavage stages of hydroids and *Acropora* implies that the same may be true in *Acropora*. However, in contrast to some hydroids, cell divisions after the eight-cell stage in *Acropora* do not appear particularly orderly, and any regular pattern of cell division quickly becomes obscured (e.g. Fig. 3 A,B). The “prawn chip” stage (Fig. 3 C-E), may be unique to corals, at least in the extreme form reached in *Acropora*, where the embryo consists of an irregularly shaped cellular bilayer (Fig. 3D). During the next stages (22 - 36 hrs), the morphogenetic movements of gastrulation result in the formation of the two germ layers, ectoderm and endoderm. In contrast to triploblasts, a third germ layer is not created. Further study is required, but the prawn chip simultaneously reduces in circumference and thickens. The edges of the thickened disc then begin to fold upward, forming a depression in one side. As this inward movement continues, cells of one of the layers become internalised and eventually lose their epithelial character and redifferentiate to form endoderm. At about 28-36 hrs (depending upon sea temperature conditions) the embryo becomes spherical with a closing pore, which we term the blastopore, although it marks the end point of gastrulation rather than its beginning.

Following closure of the blastopore, which we define as marking the end of embryonic life and the start of larval life, the larva becomes pear shaped and cilia develop (Fig. 3 G,H). An oral pore appears at the posterior end, as defined by the direction of swimming (Fig. 3I).

This pore is lined with cilia, in common with the rest of the ectoderm, but the ectodermal cells lining the oral pore are exclusively glandular in appearance, suggesting a function in extracellular digestion (Ball *et al.*, 2002). It is also during this period, from about 50 hours after spawning that new recognisable cell types begin to appear. For example, it is during the pear-shaped larval phase that neurons can first be stained with an antibody to the neurotransmitter RFamide. In the later stages of larval life there is a tendency for the planula larva to elongate from pear shaped to spindle shaped (Fig. 2) although planulae seem to be labile between the two morphologies (Fig. 3F). By the late planula stage numerous cell types have differentiated, as is clearly apparent in the trichrome-stained specimen shown in Fig. 3 (I,J). Such staining can therefore provide us with a measure of differentiation, and a context into which to fit specific cell types revealed by *in situ* hybridisation. If larval settlement cues, such as chemicals given off by coralline algae (Morse *et al.*, 1996), are not received the planula stage can survive for months in the plankton. On receipt of an appropriate settlement cue, the coral planula attaches to the substratum by the aboral end, and then contracts along the oral-aboral axis to form a flattened disc that becomes subdivided radially by mesenteries during the process of permanent settlement and metamorphosis into a juvenile coral polyp (Harrison and Wallace, 1990). In the time shortly before and immediately after settlement, there is a dramatic reorganisation of certain tissues (Vandermuelen, 1974, 1975; Harrison and Wallace, 1990), associated with metamorphosis from planula to polyp, and the start of calcification. For example, the aboral epidermis of the planula is transformed from a tall columnar epithelium into a squamous calicoblastic epithelium, which subsequently initiates and controls the development of the complex species-specific aragonite exoskeleton of the coral polyp (Harrison and Wallace, 1990).

Coral Pax Genes

Pax genes encode a large family of transcription factors with diverse functions during development of higher animals; there are nine *Pax* genes in mouse and man, and eight in *Drosophila*. *Pax* proteins are characterised by the presence of a paired domain, but most also contain a complete or partial homeodomain. Their interaction with target molecules is complex, as might be expected from their possession of multiple DNA-binding domains. The paired domain consists of two helix-turn-helix motifs (known as the PAI and RED sub-domains), each of which may be involved in DNA-binding interactions. The picture is further complicated by alternative splicing of *Pax* transcripts, which results in multiple versions of some of the nine proteins. Based on comparison of their domain structure and sequences, most of the arthropod and chordate *Pax* genes fall into four classes: the *Pax-4/6*; the *Pax-2/5/8*; the *Pax-3/7*; and *Pax-1/9*. The generally accepted view is that the common ancestor of mammals and *Drosophila* had representatives of at least these four *Pax* classes (Noll, 1993), and that these have

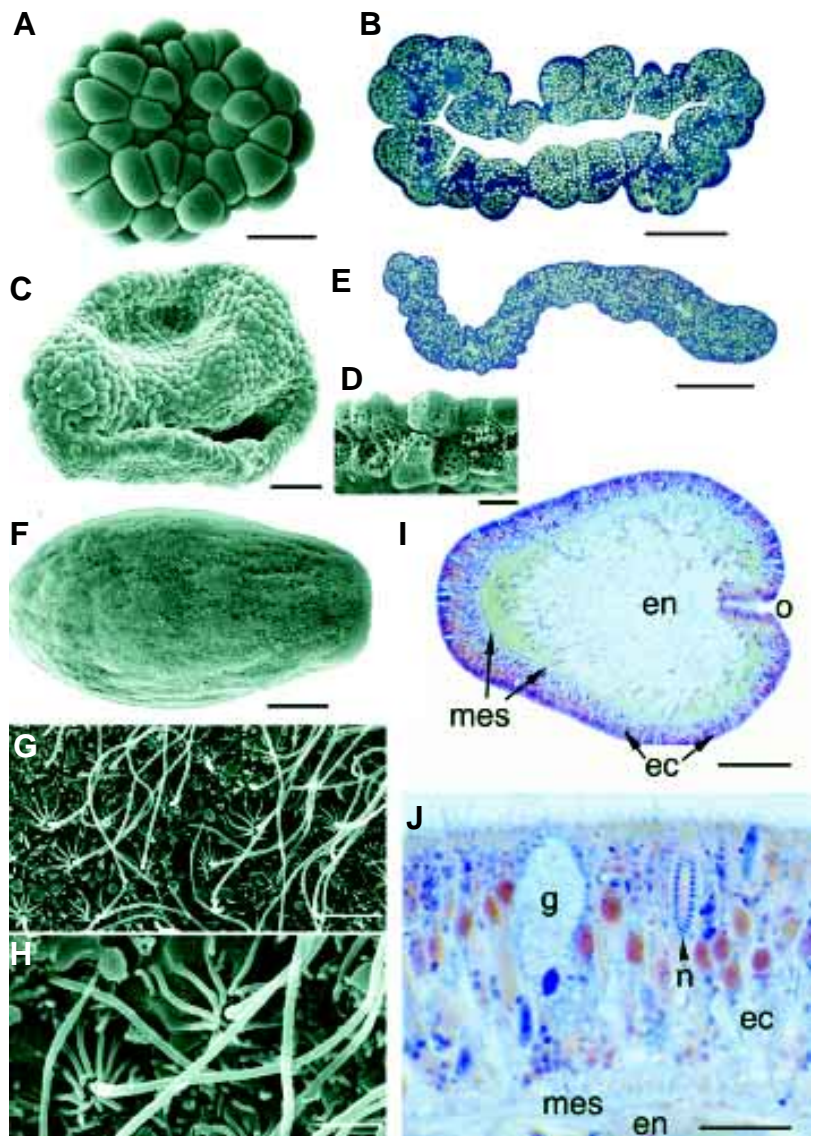


Fig. 3. Morphology and anatomy of developing embryos of *A. millepora*. (A) Embryo at approximately 9 h. (B) Stained section of a comparable embryo, showing that little intracellular differentiation has occurred. The separation between the two cell layers is artifactual, but serves to illustrate that early *Acropora* embryos consist of two layers of cells. (C) A slightly later embryo, corresponding to the interval between 11 and 13 hours in Fig. 2. (D) A high magnification scanning electron micrograph (SEM) emphasises the bilayered nature of the embryo. The cavities, which correspond to the unstained spheres in the sections shown in panels B and E, are thought to contain lipid in life. (E) Section of an embryo comparable to that shown in C. The embryo is still a bilayer and there is no sign of intracellular differentiation. (F) SEM of a planula larva. (G,H) This stage has developed external swimming cilia, each with a characteristic buttress at its base. (I,J) Trichrome staining reveals the internal structure of a planula larva. As seen in J, there is highly differentiated ectoderm (ec) on the outside of the larva, separated from the internal endoderm (en) by a thin layer of mesogloea (mes). The majority of the endodermal cells contain yolk, but among them are scattered cells of other types. This section passes through the oral pore (o), which is lined with cilia. A nematocyst (n) and gland cell (g) are labeled in J. Scale bars: A,B,C,E,F,I, 100 μ m; D, 25 μ m; G, 2.5 μ m; H, 1 μ m; J, 10 μ m.

undergone independent duplications within the two lineages since the time of divergence. As discussed in the Introduction, one way of attempting to understand the evolution of such a complex

group of genes is to investigate their presence and structure in a basal metazoan.

To date we have discovered and sequenced four *Pax* genes in the coral *Acropora* (Miller *et al.*, 2000); two of these genes (*Pax-Aam* and *Pax-Bam*) have probable orthologs in other cnidarians, but two are thus far unique to *Acropora*. Figure 4 shows results of a phylogenetic analysis of the *Acropora* paired domain sequences. This figure includes eight of the nine vertebrate *Pax* proteins (*Pax-4* is omitted as it is highly divergent), as well as most of the *Drosophila* *Pax* proteins (Twin of eyeless, a close paralog of Eyeless, is omitted). From this figure it is clear that at least some of the *Pax* gene classes in higher animals have their origins before the Cnidaria/higher Metazoa split. Of all the cnidarian *Pax* genes known to date, *Pax-Dam* is the most convincing case of orthology with a class known in higher animals. Analyses of both the homeodomain (data not shown) and paired domain (Fig. 4) put *Pax-Dam* into the *Pax-3/7* clade with high bootstrap support. Cnidarian *Pax-A* is likely to be orthologous with *Drosophila poxneuro*

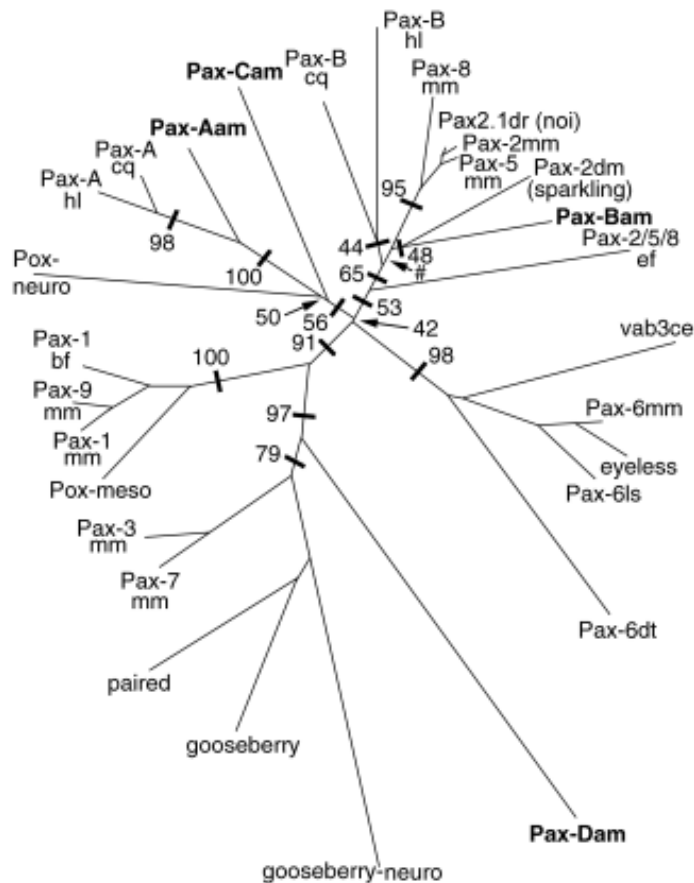


Fig. 4. Unrooted phylogram of the paired domain sequences of Pax proteins created by doing a distance analysis using the neighbor-joining method with PAUP4B2 (Swofford, 1998). Some of the coral sequences (most convincingly, that of *Pax-Dam*) group with specific classes of Pax proteins from higher animals, indicating that Pax genes were not only present in the common ancestor of corals but had already undergone a divergence. For a full discussion of this figure see Miller *et al.* (2000), from which this figure is reproduced with permission.

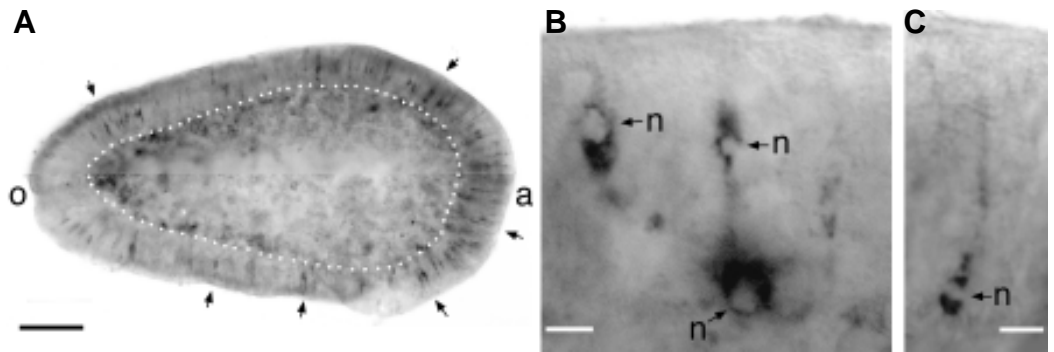
(*poxn*), a gene with no clear vertebrate counterparts and which (prior to the availability of data for cnidarians) was regarded as a highly diverged Pax-2/5/8-related gene. Although this grouping is only moderately well-supported in the distance analysis shown in Fig. 4, the rationale for regarding Pax-A as orthologous with Poxn is that they not only have the same domain structure (i.e. complete lack of a homeodomain) but also, uniquely among Pax proteins, they share the same triad of amino acid residues at three paired domain positions (42, 44 and 47). These are known to confer DNA-binding specificity in the case of Pax-2 and Pax-6 (Czerny and Busslinger, 1995).

Beyond this point, relationships between cnidarian Pax genes and the Pax classes of higher animals become less clear. The cnidarian Pax-B proteins are clearly most closely related to the Pax-2/5/8 class, although they contain complete homeodomains rather than the incomplete motifs characteristic of this class. Nevertheless, the overall level of identity in the paired domain, and the presence of octapeptide motifs closely resembling those in Pax-2/5/8 proteins, have led to the idea that cnidarian Pax-B genes resemble the evolutionary precursors of the Pax-2/5/8 class. The case of *Acropora* Pax-C is the most contentious of all; based on comparisons of the paired domains and homeodomains, as well as overall domain structure, we proposed that Pax-C represented a precursor of the Pax-4/6 class (Catmull *et al.*, 1998). Consistent with this view, Pax-C is expressed in presumed neurons during early coral development (Miller *et al.*, 2000; Fig. 5). At least at the morphological level, anthozoans show the lowest degree of nervous system complexity within the phylum Cnidaria – although (in common with all cnidarians) they show photosensitivity. However, there are no convincing eyes in any members of this class. Hence expression of Pax-C in a subset of neurons in *Acropora* is consistent with what might be expected of a Pax-6 precursor in an animal lacking true eyes. However, some cnidarians do have eyes of remarkable complexity; perhaps not surprisingly, these are at their most sophisticated in the most motile cnidarians, the box jellyfish (class Cubozoa). One problem with the notion of Pax-C as being representative of a Pax-4/6 precursor is that, if this were true, then a Pax-C gene should be involved in specifying jellyfish eyes, and to date there is no evidence that this is the case. Several groups have surveyed the Pax complements of various jellyfish (Sun *et al.*, 1997; Gröger *et al.*, 2000; Sun *et al.*, 2001), but genes related to *Pax-Cam* have not been detected. *Pax-B* was the only Pax gene detected in the hydrozoan jellyfish, *Podocoryne*, and this gene may be involved in nerve cell differentiation (Gröger *et al.*, 2000). The scyphozoan jellyfish *Chrysaora* and *Cladonema* have simple and complex lens eyes respectively; *Pax-A* has been detected in the former but not the latter (Sun *et al.*, 1997; Sun *et al.*, 2001). By comparing the domain structure of the Pax proteins between animal groups and applying phylogenetic methods of analysis, it is possible to hypothesise what the structure of the ancestral Pax protein might have been and how the various modern groups of Pax proteins might have arisen. Such scenarios can never be definitive but it quickly becomes clear that some possibilities are more likely than others. Two possible models of Pax gene evolution (Catmull *et al.*, 1998; Miller *et al.*, 2000) are shown in Fig. 6. At present, the latter of these (Model B of Miller *et al.*, 2000) seems the more likely, based on the comparative data discussed above and in the light of the report of a Pax 2/5/8 gene from a sponge (Hoshiyama *et al.*, 1998). However, testing these ideas will require

Fig. 5. *In situ* hybridisation with a Pax-Cam probe reveals that the mRNA is localised to trans-ectodermal cells resembling neurons.

(A) Low magnification photomontage shows that while expressing cells are scattered throughout the ectoderm, their density is greater at the aboral end of the pear-shaped planula larva. Arrows mark particularly strongly expressing cells, while the white dots mark the mesogloea separating ectoderm from endoderm.

(B) Two stained cells with clear nuclei lie mid-depth in the ectoderm. The basal processes of these cells appear to project to a clump of staining cells located on the mesogloea. **(C)** A monopolar cell, with a nucleus (n) which lies just above the mesogloea, projects a long extension to the surface of the ectoderm. Scale bars: A, 100 µm; B,C, 10 µm. From Miller et al. (2000) with permission.



expression data (or, more appropriately, functional data) from a range of cnidarians and other lower metazoans. Although expression and functional data are lacking for cnidarians with well-defined eyes, the available data suggest that considerable heterogeneity may exist in Pax gene use across the Cnidaria. Indeed, failure to detect Pax-C in a range of jellyfish, together with *in vitro* DNA-binding data (Sun et al., 2001; de Jong et al., unpublished data) lead us now to question the notion of a simple correspondence between Pax-B and Pax-C in cnidarians and the Pax-2/5/8 and Pax-4/6 classes in higher animals.

Nuclear Receptor Genes

The nuclear receptor genes code for another class of transcription factors of major developmental importance. The most familiar, and probably the most studied, members of this class are the vertebrate steroid hormones. Nuclear receptor proteins contain two characteristic conserved domains, a DNA binding domain (DBD) and a ligand binding domain (LBD). With the exception of the sponges, nuclear receptors are found throughout the Metazoa (Escriva et al., 1997). The nuclear receptor gene superfamily can be divided into six subfamilies defined by phylogenetic analyses, and this system of classification is used as the basis for a unified nomenclature system (Nuclear Receptors Nomenclature Committee, 1999)

Classically, nuclear receptors are ligand modulated transcription factors. Upon ligand binding they undergo a conformational change which leads to transcriptional activation of target genes. Many identified nuclear receptors, however, have no known ligand; these are the so-called orphan receptors. It is likely that ligands will be discovered for many orphan receptors, as has already happened in some cases (for reviews see Blumberg and Evans, 1998; Giguere, 1999), but other orphan receptors may function without a ligand.

Previous to our studies in *Acropora* (Grasso et al., 2001), five distinct cnidarian PCR products were known, apparently belonging to the three gene classes COUP-TF, RXR, and FTZ-F1. They came from the hydrozoan, *Hydra* (one) and the anthozoan, *Anemonia* (four) (Escriva et al., 1997). This work was soon followed by the complete cDNA sequence of an RXR from the cubozoan jellyfish, *Tripedalia cystophora* (Kostrouch et al., 1998). The highly conserved sequence of the DNA binding domain of

nuclear receptors allowed us to design primers to search the *Acropora* genome, using the polymerase chain reaction (PCR), for all of the classes of nuclear receptors which have been described from higher animals (Grasso et al., 2001). Eight distinct PCR products were produced from *Acropora* planula cDNA using primers designed to amplify products corresponding to members of nuclear receptor subfamily 2. These same eight products were

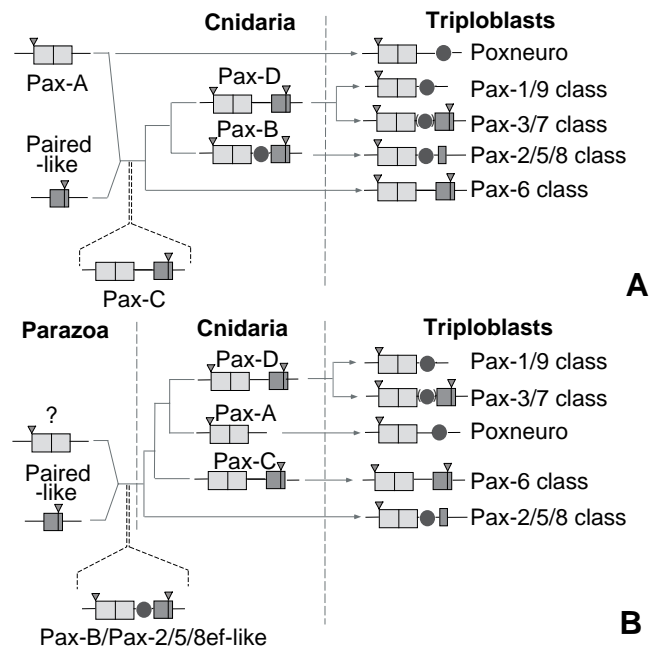


Fig. 6. Alternative models for the evolution of the various Pax gene classes. A and B represent alternative schemes, first proposed in Catmull et al. (1998) and Miller et al. (2000). The only substantial difference between these is that in (A) a Pax-C-like gene is viewed as ancestral to all of the vertebrate Pax classes, whereas in (B) a Pax-B-like gene is basal. The occurrence of a sponge gene that appears to be most closely related to the Pax-B type (Hosiyama et al., 1998) favors scheme B. However, note that *in vivo* and *in vitro* DNA-binding experiments (Sun et al., 2001; de Jong et al., unpublished observations) lead us now to question the notion of a simple correspondence between Pax-B and Pax-C in cnidarians and the Pax-2/5/8 and Pax-4/6 classes in higher animals. From Miller et al. (2000) with permission.

obtained from genomic DNA along with two additional products. Primers targeting other nuclear receptor subfamilies either failed to produce a product or produced a subset of these ten. These results indicate that *Acropora* has more than twice the number of nuclear receptors previously recognised from a single cnidarian species (four from the anemone, *Anemonia*).

cDNAs corresponding to seven of the PCR products were sequenced from an *Acropora* planula (Fig. 2, 96 hours) library. At least three of these contain complete open reading frames, raising the number of complete cnidarian nuclear receptor coding sequences from one to four. The data from *Acropora* indicate that the common ancestor of cnidarians and the rest of the Metazoa possessed at least three nuclear receptor genes, which gave rise in both lineages to the Tailless, COUPTF, and HNF4 classes of nuclear receptors. The presence of a clear RXR ortholog in the cubozoan, *Tripedalia*, suggests that it too was present in the common ancestor. Thus far, no corresponding gene has been found in *Acropora*, but candidate PCR products have been generated and the corresponding cDNA clones may reveal it. Thus, although some of the better known members of the nuclear receptor family, such as the steroid receptors, appear to have evolved exclusively in vertebrates, nuclear receptors are also an ancient class of molecules that had diversified before the separation of the Cnidaria from the ancestral metazoan stock.

Studies on *Acropora* also provide some insight into evolution within the nuclear receptor family. Figure 7 is a maximum likelihood tree showing the relationships between the *Acropora* nuclear receptors and a representative sample, from the database, of nuclear receptors which can be aligned. According to the Nuclear Receptors Nomenclature Committee (1999) there are six nuclear receptor subfamilies and at least one member of each subfamily is included in the analysis. All but two of the *Acropora* nuclear receptors clearly fall into subfamily 2. The two which do not, AmNR2 and AmNR6, are closely related, very difficult to place anywhere within the phylogeny of nuclear receptors, and may represent an ancestral gene which has undergone extensive divergence in the coral lineage. Named groups within subfamily 2 are indicated on the tree. AmNR4, and AmNR8 are highly similar, and probably represent a coral-, or cnidarian- specific gene duplication. They group with AmNR5 and are most closely related to the TR2/4 group. Thus, the results from *Acropora* are consistent with the idea that the common ancestor of all nuclear receptors most resembled those in subfamily 2.

All unequivocal orthologs of the cnidarian nuclear receptors in other phyla are classified as orphan receptors. This finding supports the suggestion that the ancestral nuclear receptor was without a ligand (Escriva *et al.*, 1997, 2000).

Hox-like and Other Homeobox Genes

Cnidarians were an obvious group on which to test the hypothesis that there was a set of so called "zootype genes", common to all animals, which were expressed in a characteristic order along the anterior/posterior (A/P) axis (Slack *et al.*, 1993). At the core of this group of zootype genes were the Hox genes, homeobox-containing genes that are expressed in just this way along the A/P axes of insects and vertebrates. Early attempts to clone Hox-related genes from cnidarians were successful (*Hydractinia* and *Eleutheria*, Schierwater *et al.*, 1991; *Acropora*, Miller and Miles 1993) and it seemed only a matter of time before others in the cluster, albeit perhaps a smaller set, would be cloned. This seemed particularly likely after Miller and Miles (1993) established that a Hox-like gene and an *even-skipped*-like gene were physically linked in *Acropora*, as they are in vertebrates (Faiella *et al.*, 1991). Most of the cnidarian literature of the 1990's assumed the existence of cnidarian Hox genes and much time was spent debating how many Hox-related genes the ancestral cnidarian had inherited. However, although many more cnidarian homeobox genes have indeed been cloned (for review see Gauchat *et al.*, 2000), the passage of time has led to more, rather than less, uncertainty about the existence of cnidarian Hox genes. There are several reasons for this uncertainty. The first has to do with the definition of a Hox gene. To qualify as a Hox gene a gene must obviously contain a homeobox similar in sequence to a specific Hox gene in the higher Metazoa. But even undoubtedly orthologous insect Hox genes may have little similarity to each other outside of the homeobox, and the homeobox is relatively small, so how much notice should one take of small differences in sequence? When comparing supposedly orthologous genes in two insects the expression patterns of the two genes can often be used to confirm whether the genes are indeed orthologs. However, when comparing across phyla the use of expression becomes much more difficult. For example, in cnidarians there is even uncertainty as to what is the anterior-posterior axis and how it should be defined (e.g. Hayward *et*

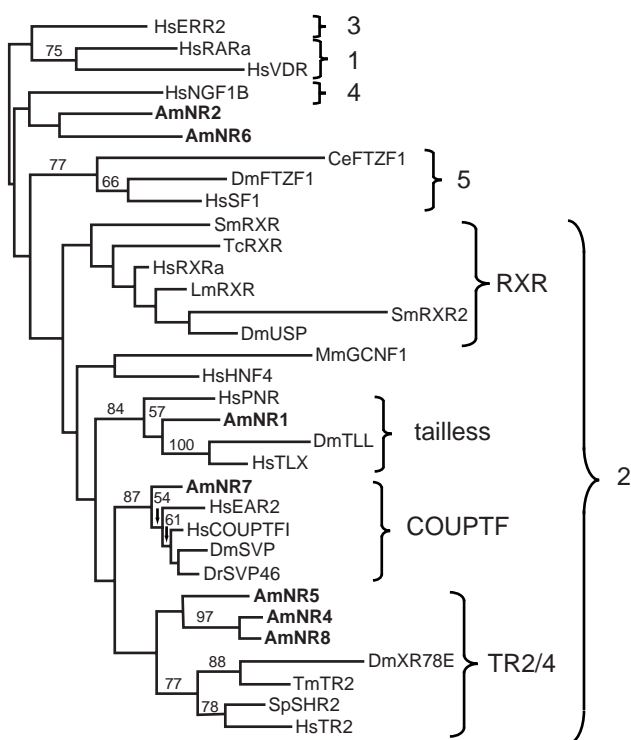


Fig. 7. Maximum likelihood tree showing the relationship of *Acropora* nuclear receptors with other nuclear receptors. Bootstrap scores above 50% are shown. Numbers 1-5 indicate the nuclear receptor subfamilies defined by the nomenclature committee (Nuclear Receptors Nomenclature Committee, 1999). Within subfamily 2 the RXR, Tailless, COUPTF and TR2/4 groups are labeled. On this tree, the single subfamily 6 representative, MmGCNF1, groups with subfamily 2 members. Modified from Grasso *et al.* (2001).

al., 2001). If axes defined by swimming direction and molecular markers turn out to provide contradictory results, then which parameter should be used to define the axis? Since the early days of the zootype hypothesis, it has also become clear that there exist higher metazoan genes, for example *Gsx* and its orthologs, which are clearly Hox genes on the basis of their structure but are orphans (i.e. not a part of a Hox gene cluster). Holland and his collaborators (Brooke *et al.*, 1998) proposed that the reason for this apparent anomaly was that an early duplication of the precursor of the Hox cluster resulted in a parallel Para-Hox cluster. Three genes were proposed as members of the Para-Hox cluster in amphioxus, and their expression patterns were indeed consistent with such a proposal. However, no similar clusters have been reported in other organisms since that time, placing the generality of this hypothesis in question.

A second problem for those wishing to argue in favour of Hox genes in Cnidaria is the extent to which a cluster of such genes is necessary in order for any gene to be classified as a Hox gene. Even some of those who believe that cnidarians do have Hox genes accept the argument that a true Hox gene should be part of a cluster. For example, Ferrier and Holland (2001) support their contention that cnidarians have Hox genes by citing an unpublished report that has not since been confirmed.

While a physical linkage between Hox-like genes in cnidarians cannot yet be ruled out, several studies have thus far failed to find such a linkage. The only promising lead has been the previously mentioned link between a Hox-like gene and an *even-skipped*-like gene, first reported in *Acropora* (Miller and Miles, 1993) and since confirmed in another anthozoan, *Nematostella* (Finnerty and Martindale, 1999).

Another factor that has weakened the argument for Hox genes in the Cnidaria is the realisation that, with the great increase in the amount of sequence data available, genes once thought to be closely related phylogenetically now appear much less so. The gene *cnox-2* (CNidarian homeobox gene 2) is a good case in point. It is indeed a Hox-like gene, but with the appearance of additional comparative sequence data, it groups more closely with members of the *Gsx* family of homeobox genes than with the true Hox genes (Finnerty and Martindale, 1999; Gauchat *et al.*, 2000; Hayward *et al.*, 2001). Thus, while it is still uncertain whether there is physical linkage between at least some Hox-like genes in Cnidaria, currently it appears to us more likely that no linkage will be found. Therefore, at least some of the cnidarian Hox-related genes can probably best be viewed as corresponding to "proto-Hox" genes (Schierwater and DeSalle, 2001).

In spite of apparently lacking a Hox gene cluster, cnidarians do have a wide variety of homeobox genes. For example, Gauchat *et al.* (2000) recognise thirteen different classes of homeobox genes within the Antennapedia superfamily in cnidarians and the number of such genes is still growing. In *Acropora*, representatives of nine of these classes have been identified.

Gene Discovery in *Acropora*— a Preliminary EST Study

One clear message from the previous sections should be that a surprising diversity of genes has been identified in *Acropora*— at least four genes encoding Pax proteins, ten encoding nuclear receptors, and a range of other genes encoding a considerable diversity of Antennapedia superclass homeodomain proteins. These

findings hint at cryptic complexity in cnidarians; the assumption has been that the specification of a single body axis, two body layers composed of relatively few cell types and a "simple" nerve net would require only a small fraction of the genes used (for example) to specify the much more complex body plan of *Drosophila*. However, a preliminary EST project, together with the work on specific gene families described above, challenges this and several other assumptions about the evolution of developmental control genes within the animal kingdom.

In addition to the issue of just how many genes are likely to be present in *Acropora*, the EST results surprised us in two other ways. The first surprise was that, despite the fact that the Cnidaria are equally diverged from the chordate, nematode and arthropod lineages, coral genes frequently match significantly better to their chordate orthologs than to *Drosophila* or *Caenorhabditis* genes. Even more surprisingly, analyses of a subset of the EST data also show that, in three-way comparisons, coral/human distances often turn out to be closer than fly/human distances. The second surprise was that a number of genes previously thought to be vertebrate-specific are present in *Acropora*. Genes that have been characterised in vertebrates but are clearly absent from *Drosophila* and *Caenorhabditis* have been assumed to have recent origins, but the coral data indicate that there are a number of cases where this assumption was incorrect. Although the analyses are not yet complete, the implications are clear - with the exception of paralogs arising through duplication events, far fewer genes are likely to have been vertebrate innovations than has been assumed to date. Instead, gene loss is likely to have been more extensive in both *Drosophila* and *Caenorhabditis* than was previously suspected.

Our rationalisation of these findings is that, of the genes central to the development of higher animals, many more are likely to predate the Cnidaria/higher Metazoa split than has previously been assumed; *Acropora* has inherited a common set of genes and patterning processes that were present in the common metazoan ancestor, but has perhaps not explored the possibilities that these genes offer to the extent seen in bilateral animals. In other words, members of the genus *Acropora* may have essentially 'frozen' the genetic possibilities they inherited whereas, through cooption, gene duplications, and changes in both regulatory and coding sequences, higher animals have more fully exploited the range of evolutionary options offered by these genes.

The basal phylogenetic position of the Anthozoa, and the surprising extent to which key genes appear to have been conserved between cnidarians and chordates, implies that further research on *Acropora* is likely to provide unique perspectives on common molecular principles of animal development. If analyses of the full EST data set support our preliminary findings, then we anticipate a great deal more interest in *Acropora* as a model system from the developmental genetics community.

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