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EST Analysis of the Cnidarian *Acropora millepora* Reveals Extensive Gene Loss and Rapid Sequence Divergence in the Model Invertebrates

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Summary

A significant proportion of mammalian genes are not represented in the genomes of *Drosophila*, *Caenorhabditis* or *Saccharomyces*, and many of these are assumed to have been vertebrate innovations. To test this assumption, we conducted a preliminary EST project on the anthozoan cnidarian, *Acropora millepora*, a basal metazoan. More than 10% of the *Acropora* ESTs with strong metazoan matches to the databases had clear human homologs but were not represented in the *Drosophila* or *Caenorhabditis* genomes; this category includes a surprising diversity of transcription factors and metabolic proteins that were previously assumed to be restricted to vertebrates. Consistent with higher rates of divergence in the model invertebrates, three-way comparisons show that most *Acropora* ESTs match human sequences much more strongly than they do any *Drosophila* or *Caenorhabditis* sequence. Gene loss has thus been much more extensive in the model invertebrate lineages than previously assumed and, as a consequence, some genes formerly thought to be vertebrate inventions must have been present in the common metazoan ancestor. The complexity of the *Acropora* genome is paradoxical, given that this organism contains apparently few tissue types and the simplest extant nervous system consisting of a morphologically homogeneous nerve net.

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Results and Discussion

Our understanding of metazoan genome evolution is based on a small number of complete genome sequences and large EST datasets that represent only a few complex animals. Of necessity, therefore, deductions about the evolutionary origins and structures of human genes are largely based on comparisons with the genomes of the insects *Drosophila melanogaster* and *Anopheles gambiae*, the nematode *Caenorhabditis elegans*, and the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The study of the evolution of developmental genes has identified some spectacular examples of conservation of developmental programs, particularly between *D. melanogaster* and vertebrates. However, a significant number of *D. melanogaster* and *C. elegans* genes are highly modified, and the extent of gene loss in these organisms is unknown [1–3]. Substantial differences between the *D. melanogaster* and *C. elegans* genomes, together with the fact that the nematodes and arthropods are now generally regarded as more closely related than was previously the case [4, 5] (although see also [6]), imply that comparisons based only on these organisms may give a misleading view of the ancestral metazoan.

In terms of understanding the evolution of metazoan genetic and developmental complexity, the Cnidaria are likely to be critically important, as this phylum is regarded as the sister group to the Bilateria [7]. The limited available data suggest that cnidarian genes may not only reflect ancestral intron/exon structures [e.g., 8, 9], but also highlight gaps in our understanding of animal genome evolution. For example, our analysis of DPP pathway genes in *A. millepora* revealed a remarkable level of conservation between these genes and their vertebrate, rather than their invertebrate, counterparts [10, 11]. Jellyfish (*Podocoryne carnea*) equivalents of the myogenic genes *Brachyury*, *Mef2*, and *Snail* are also more similar to their vertebrate homologs than to their *D. melanogaster* and *C. elegans* counterparts [12].

To obtain a broader perspective on patterns of gene evolution, we conducted a limited EST analysis on the coral *Acropora millepora*. *A. millepora* is a member of the basal cnidarian class, the Anthozoa, making it an appropriate cnidarian for comparative purposes [13]. A 96 hr planula larva stage cDNA library for *A. millepora* [14] was normalized prior to sequencing individual clones. cDNAs were amplified using vector primers, denatured at 100°C for 5 min, and then allowed to reassociate at 65°C for 24 hr prior to hydroxyapatite chromatography at 60°C in 0.12 M phosphate buffer (pH 6.8). After concentration, the eluate was used as template in PCR and products cloned into pGEM-T easy (Promega). 3024 independent clones were isolated, and 2592 single-pass sequences were obtained using the T3 primer. Using the phred/phrap package (Brent Ewing and David Gordon, University of Washington), clone sequences were filtered for vector contamination and quality. This resulted in 632 sequences being discarded. The remaining se-

quences were grouped into 1376 EST clusters using BLASTN [15]. 999 ESTs were present as unique sequences, while 961 clones formed 377 clusters of two or more clones, which were treated together in subsequent analyses. The EST sequences were compared to the GenPept database (November 7, 2002) using the BlastX program [15] with a Blast E-statistic acceptance threshold of 10^{-6}. In total, 492 ESTs matched entries in the GenPept database with E-statistics ranging from 10^{-6} to 10^{-137}. The EST sequences are available at http://cbis.anu.edu.au/coral/.

Gene Loss in *Drosophila* and *Caenorhabditis*

The most surprising implication of the *Acropora* dataset is that extensive gene loss has occurred in *Drosophila* and *Caenorhabditis*—a substantial number of the coral ESTs (53 clusters; 11% of hits to any organism) appeared to have a human homolog but no counterpart in the fly or worm. A much smaller number (five clusters; 1% of hits to any organism) of the sequences gave significant matches only with fly or worm genes. Table 1 lists those *A. millepora* ESTs with nominal homologs in only either *H. sapiens* or the model invertebrates. To assess whether these genes arose in, or predate, the metazoan lineage, the *A. millepora* sequences were used to search for homologous sequences in representatives of other kingdoms. Of the 58 sequences listed in Table 1, all but six lack identifiable homologs in other kingdoms, suggesting that these genes are likely to have been metazoan innovations that have subsequently been lost from at least some (metazoan) lineages.

The absence of clear *D. melanogaster* or *C. elegans* homologs of many genes represented in the coral and human genomes thus reflects secondary gene loss in the lineages that gave rise to these model invertebrates. In at least some cases, however, these genes have been assumed to be vertebrate innovations. Examples include Churchill (AmEP01258; [16]) and Tumorhead (AmEP00036; [17]), two genes involved in the regulation of early neural development in vertebrates. Such gene losses are not confined to specific functional classes, although metabolic/structural proteins and transcription factors account for most of those identified. The scale of gene loss—53 genes missing from the genomes of both fly and worm identified in this analysis of 1376 EST clusters—implies that a major fraction of nominally vertebrate-specific genes were, in fact, present in the common metazoan ancestor. Decreasing the matching stringency increases the proportion of nominal vertebrate-only matches in the *Acropora* dataset (data not shown; note also that there is a corresponding decrease in confidence in the *Acropora*/human match). Broadening the search to consider matches with other vertebrates likewise identifies additional cases of nominally vertebrate-specific genes in the coral EST dataset; for example, a clear match with snake venom phospholipase A2 is represented both in the *Acropora* dataset ($1e^{-14}$) and in the jellyfish *Cyanea* [18]. No other systematic attempts have been made to assess the proportion of genes shared between cnidarians and vertebrates that are missing from *Drosophila* and *Caenorhabditis*. However, searching for specific candidates amongst the limited data available for other cnidarians allowed us to identify a number of other genes in this category; for example, homologs of the Dickkopf proteins (mostly Wnt antagonists) and p8 transcription factors can be found amongst the available *Cyanea capillata* ESTs [18], while a clear homolog of the human Bardet-Biedl syndrome 4 gene is present in *Hydra magnipapillata* (database accession number AAO72330). Nearly one-sixth of genes identified in the urochordate *Ciona* lack a clear *Drosophila* or *Caenorhabditis* homolog but are represented in the *Fugu* or human genomes [19]. Whereas these genes have been assumed exclusively to represent chordate- (or deuterostome-) specific innovations [19], the cnidarian data imply that this interpretation is probably incorrect and that although some chordate-specific genes undoubtedly exist, the number is likely to be much smaller than has been assumed to date. Consistent with this, most, but not all, of the genes listed in Table 1 as uniquely shared between *Acropora* and man are present in *Ciona* and would therefore be amongst the 2570 genes previously considered as likely to be chordate specific [19]. Hence, many genes assumed to have much later evolutionary origins are likely to have been present in the common metazoan ancestor and have been lost from the fly and worm. In some respects, this pattern of gene loss in metazoans resembles that reported for fungi; *Saccharomyces cerevisiae* appears to have lost about 300 genes since divergence with *Schizosaccharomyces pombe* [20, 21]. Although the true extent of gene loss from the model invertebrates will only become clear after comprehensive analyses of a range of non-standard animals, the complete genomic sequence of an anthozoan such as *Acropora* or *Nematostella* is likely to identify many genes previously assumed to be vertebrate innovations.

The Derived Nature of Many Other *Drosophila* and *Caenorhabditis* Genes

A more subtle revelation of the *Acropora* EST dataset is the extent to which coral sequences resemble human genes rather than the corresponding *Drosophila* and *Caenorhabditis* sequences; in comparisons against the entire database, the majority of the coral ESTs show much higher similarity to vertebrate sequences than to any invertebrate sequences (Figure 1A). For clarity, we conducted comparative analyses to *H. sapiens* and the model invertebrates *D. melanogaster* and *C. elegans*, and for each, near complete genome sequences are available. Of the 455 ESTs identified with clear matches in at least one of these three metazoans, only a small proportion (33 or 7%) showed $>10^3$-fold more significant similarity to fly and/or worm sequences, as measured by BLAST E-value, while 165 (36%) showed $>10^3$-fold more significant similarity to human sequences (Figure 1B). These results confirm a trend in closer relatedness between coral and vertebrate sequences (than between coral and fly or worm sequences) that we have observed in previous studies [10, 11]. The same general trend has been observed with specific proteins from some other cnidarians (see, for example, [12]). The general trend toward higher overall similarity is not confined to specific functional classes of genes. It is true of genes that encode

Table 1. *A. millepora* ESTs, Which in Comparisons with *H. sapiens*, *D. melanogaster*, and *C. elegans*, Are Uniquely Shared with Either Man Or the Model Invertebrates

Uniquely Shared with Man:			
AmEP00013	Similar to RIKEN 1700003M02 (unknown function)	BC035083	6e-19
AmEP00017	Uncharacterized WD, SAM and U box domain protein	BC029520	2e-16
AmEP00036	Tumorhead (*Xenopus*) homolog	AK055726	6e-8
AmEP00100	Cystatin B (stefin B)	BC010532	2e-21
AmEP00144	MBT-related	AL110249	8e-8
AmEP00176	Unknown function (contains OST3/OST6 domain)	AF161425	7e-16
AmEP00191	t-sec1 (mouse) homolog	BC028119	3e-12
AmEP00195	L-arginine:glycine amidinotransferase	X86401	6e-57
AmEP00227	Agmatinase (agmatine ureohydrolase)	AY057097	1e-44
AmEP00233	Unknown function	AK098840	7e-21
AmEP00235	M phase phosphoprotein 6	BC031017	4e-20
AmEP00265	Alpha 1 type III collagen	BC028178	5e-18
AmEP00274	Cytochrome c oxidase subunit VIIc	BC007498	2e-8
AmEP00280	Dolichyl-phosphate mannosyltransferase polypeptide 3	BC032223	8e-13
AmEP00301	Retinol dehydrogenase 8 (all-trans)	AK024022	1e-6
AmEP00320	Chromosome 22 ORF 23 UPF0193/Pfam05250/EVG1	BC031998	2e-8
AmEP00322	Alpha X integrin (Cd11c/leukocyte adhesion p150)	Y00093	7e-7
AmEP00346	PCAF associated factor 65 alpha (TAF6L)	AF069735	6e-15
AmEP00352	Unknown function	BC031107	2e-11
AmEP00422	Hypothetical protein FLJ90575 (unknown function)	AK075056	3e-32
AmEP00443	Subtilisin/kexin isozyme SKI1-related	AL133583	1e-7
AmEP00451	Zinc finger protein 294	AB018257	4e-7
AmEP00516	HMG protein HMGX1	AF146222	6e-18
AmEP00522	PRTD-NY2 (contains RGS domain)	AY009106	2e-11
AmEP00564	HGF activator precursor	D14012	4e-8
AmEP00589	Microtubule actin crosslinking factor 1 isoform 4	AB007934	2e-12
AmEP00622	KPL2 (rat)-related	AK027230	4e-12
AmEP00640	Adenylate kinase 5-related	AK090967	2e-8
AmEP00671	KNP-I/ES1 (zebrafish) homolog	BC003587	4e-24
AmEP00727	N-methylpurine DNA glycosylase	AF499437	4e-31
AmEP00759	Glutathione S-transferase Mu4	X68677	4e-16
AmEP00762	Zinc finger protein 291	AF242528	1e-19
AmEP00772	Microsomal glutathione S-transferase 3	BC005964	6e-29
AmEP00781	Methyl-CpG binding protein 2	BC032638	4e-20
AmEP00808	Cysteine rich FGF receptor	U28811	1e-7
AmEP00831	Unknown function	AK094715	4e-21
AmEP00843	Similar to Ts mitochondrial translation EF	BC022862	5e-11
AmEP00909	NYD-SP14 (unknown function)	BC024193	9e-13
AmEP00926	Hypothetical protein FLJ21977 (ELMO-related)	BC010991	8e-10
AmEP00972	Chromosome 4 ORF45	AL834487	2e-18
AmEP00986	Acid phosphatase type 5	J04430	6e-10
AmEP00998	Unknown function	AK092816	4e-18
AmEP01004	Similar to KIAA0543 protein (unknown function)	AC004877	5e-7
AmEP01027	Neuronal pentraxin 1	U61849	2e-9
AmEP01064	Serine dehydratase-related	BC009849	2e-15
AmEP01203	Inositol polyphosphate 4-phosphatase	U26398	7e-7
AmEP01241	Unknown function	BC004228	8e-15
AmEP01258	Churchill (mouse) homolog	BC020550	1e-27
AmEP01267	Spasmolytic polypeptide (trefoil domain containing)	X51698	5e-14
AmEP01274	Related to retinitis pigmentosa 9	BC025928	7e-26
AmEP01283	Kinectin 1	Z22551	4e-11
AmEP01304	Thymosin beta-10	M92383	1e-10
AmEP01317	Unknown function (possible Smc domain)	AB040938	2e-22
Uniquely Shared with *Drosophila* or *Caenorhabditis*:			
AmEP00030	*Drosophila* CG3698 (unknown function)	NM_141000	9e-15
AmEP00080	*Drosophila* CG10933 (unknown function; SH3 domains)	NM_137407	1e-12
AmEP00223	*Drosophila* Pao retrotransposon peptidase	U23420	4e-9
AmEP01190	*Caenorhabditis* (putative secreted or extracellular)	NM_077448	1e-7
AmEP01217	*Drosophila* CG7102 (unknown function; TLDc domain)	AY118856	3e-9

The left column gives the *A. millepora* EST cluster number. The best match on BlastX comparisons with the database are given, along with the corresponding Genbank database identifier and the e value. To confirm that those sequences identified are lineage restricted, secondary database comparisons were carried out. Human (or fly or worm) proteins matching the coral ESTs were themselves used to search the databases (using BlastP) followed by manual inspection of the results.

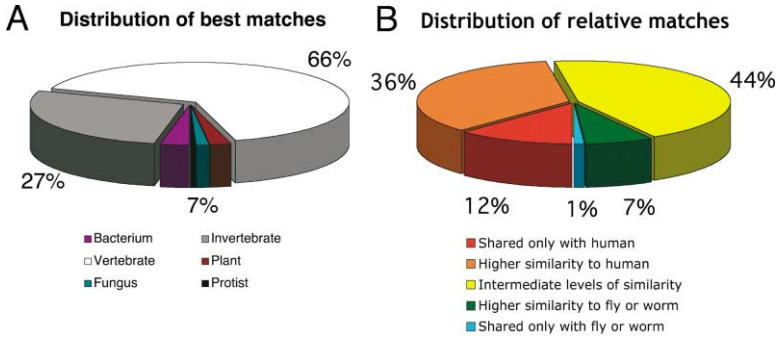

Figure 1. Summary of *Acropora* EST Sequence Comparisons

(A) Distribution of ESTs giving a best match to specific organism classes. In this case, the proportions of the 492 EST clusters giving strong database matches are indicated.

(B) Distribution of relative matches amongst the representative invertebrates (*D. melanogaster* and *C. elegans*) and *H sapiens*. In this case, the dataset consisted of the 397 ESTs with both fly/worm and human matches and the 58 ESTs that had only either fly/worm or human matches (see Table 1 for a breakdown of the latter category). Note that the phrase ">Higher similarity..." refers to $>10^3$-fold lower BLAST-E values (see text). Although a significant number of matches were with bacterial sequences, these are unlikely to represent contamination as the library was generated from polyA$^+$ mRNA [14], and the best matches were with taxonomically diverse bacteria.

transcription factors, such as DC6/E(y)2 (AmEP01369), Hex (AmEP00044), Y box transcription factor (AmEP00530) and Snail (AmEP00427), as well as for components of cellular signaling pathways, such as DPP, Smads and Delta (AmEP00563), and for housekeeping proteins such as methyl CpG binding protein (AmEP00781) and WASp (AmEP00290). In many cases, the highest levels of identity were between coral and human sequences, with the worm and fly typically showing higher levels of divergence. Figure 2 shows phylogenetic analyses of some representative coral sequences, illustrating the general trend of much higher levels of identity with human than with fly or worm sequences.

Although a close relationship between coral and human sequences is superficially surprising given that the cnidarian and bilaterian lineages are thought to have diverged in deep Precambrian time [22], this is largely a consequence of the relative pace of change in the model invertebrates. The greater divergence in *D. melanogaster* and *C. elegans* sequences is unlikely to reflect uniform rates of change over long time periods; rather, rapid genome change is likely to have occurred recently (and probably independently) in these organisms and be associated with intense selection for small genome size, rapid developmental rates, and the highly specialized lifestyles of the fly and worm. Although *D. melanogaster* had the previously reported fastest rate of sequence change, the genes of *C. elegans* are evolving even faster [23, 24], and genome rearrangements are occurring approximately four times faster in the worm than in the fly [24]. Typically long branch lengths in phylogenetic analyses (see Figure 2) support the idea that many *D. melanogaster* sequences are highly derived relative to their coral and human counterparts. This is also true of many *C. elegans* sequences [23, 25]. If this hypothesis is correct, we might expect sequences from more "primitive," and so less-derived, protostomes to be more closely related to the coral/human gene set; Hox data for the ribbonworm *Lineus* are consistent with this hypothesis [26]. Comparisons with lophotrochozoans and with the cephalochordate amphioxus will be particularly informative; however, at present these are poorly represented in the databases. Comparisons with the urochordate *Ciona* [19] emphasize the derived position of the model invertebrates and, although only limited comparative data are available for representatives of the more basal insect orders, these often also dramatically support the derived position of *D. melanogaster*. For example, although *D. melanogaster* does not carry out standard CpG methylation and lacks typical MBD proteins [27], more primitive insects such as the cricket *Acheta* are more vertebrate-like in both respects (see Figure 2C; [28]). Similarly, comparisons of retinoic acid receptor ligand binding domains (RXR LBDs) indicate that tick, crab, and grasshopper (*Locusta migratoria*) sequences are more similar to their vertebrate orthologs than to the *D. melanogaster* LBD or those of other holometabolous insects [29].

Genetic Complexity and the Common Ancestor

A third implication of our analyses is that at least at the level of gene complement, the ancestral metazoan is likely to have been much more complex than was previously imagined. For example, the *A. millepora* EST dataset contains homologs of many bilaterian genes whose specialized functions are associated with highly differentiated nervous systems. These include genes with vertebrate, but no known invertebrate, counterparts; e.g., those that encode photoreceptor all-*trans*-retinol dehydrogenase (AmEP00301), Churchill, and Tumorhead. They also include more generally conserved homologs of genes that encode Frequenin, Homer 2d, Glia maturation factor b, and Notch pathway components. This complexity is particularly surprising given the morphological simplicity of the coral nervous system (anthozoans have the simplest extant nervous systems—morphologically homogeneous nerve nets) and the absence of recognizable photoreceptors. Nevertheless, coral larvae display phototactic behavior [30] and the Pax-6-related gene *PaxC* is expressed in a subset of *A. millepora* presumed neurons [31]. The detection of ESTs matching *hex* and *snail*, genes that play key roles in endoderm and mesoderm patterning in triploblastic animals, supports the renewed interest in the nature of the cnidarian primary tissue layers [12]. At the very least, these findings provide a strong argument for developing a much better understanding of cnidarian developmental mechanisms, if we are to understand the origins of these mechanisms.

Conclusions

Our preliminary survey of the expressed sequences of planula stage *Acropora millepora* appears to turn upside

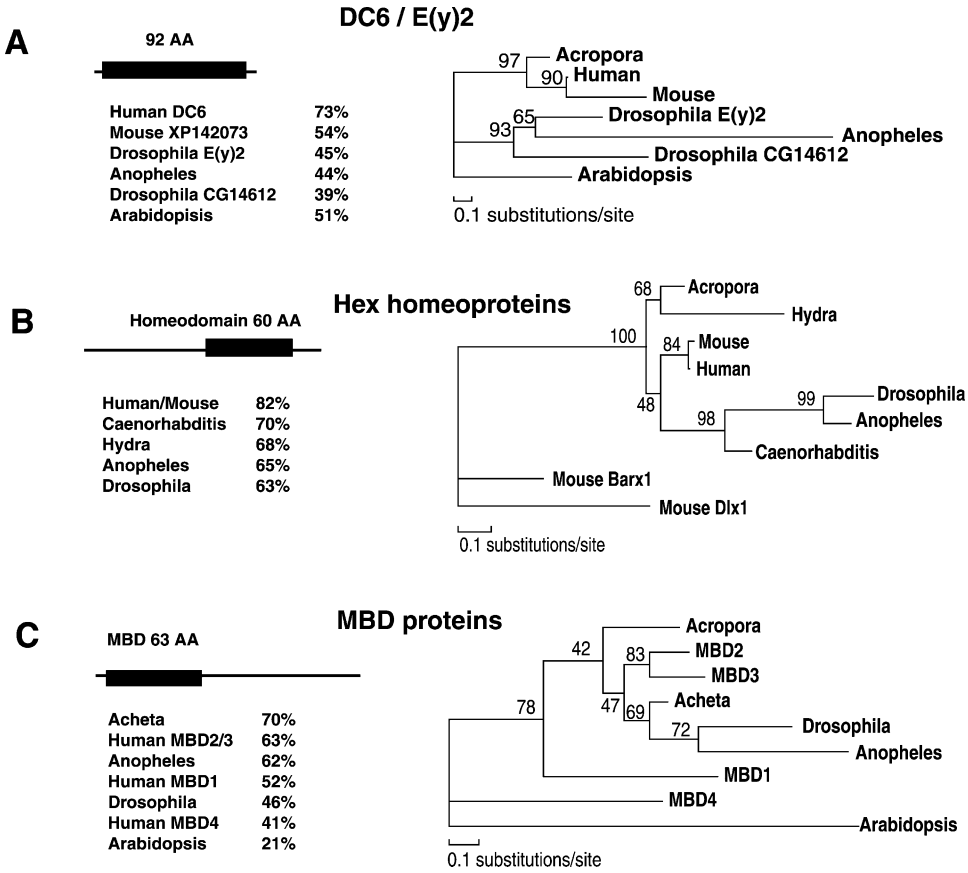

Figure 2. Comparison of Coral Sequences Indicates Higher Levels of Identity with Human Than with *D. melanogaster* or *C. elegans* Sequences

Maximum likelihood phylogenetic analyses of the conserved protein domains were conducted in MolPhy version 2.3 (Institute of Statistical Mathematics, Tokyo) [32] using the Dayhoff substitution matrix and local rearrangement search mode. In each case, the percent identity with the *A. millepora* sequence is indicated below the protein schematic, and the figures on branches in the phylogenetic trees indicate percent of 1000 (ML) bootstrap replicates supporting the topology shown.

(A) *A. millepora* EST (AmEP01369) shows high levels of identity with human and mouse DC6 (dendritic cell protein 6), whereas the *D. melanogaster* enhancer of yellow [E(y)2] protein has lower identity with both the *A. millepora* and vertebrate sequences. *C. elegans* does not appear to have a member of this orthologous group; the most closely related sequence is a hypothetical protein H05L14.2/Z99772/Z75533, which has only a partial match.

(B) The *A. millepora* Hex homeodomain has higher levels of identity with its vertebrate rather than with its *Hydra* homologs. However, in ML phylogenetic analyses, the cnidarian sequences form a monophyletic group, showing that the hydra sequence is highly derived.

(C) *A. millepora* EST AmEP00781 encodes a typical methyl-CpG binding domain most closely related to vertebrate MBD1 and MBD2. The corresponding *D. melanogaster* protein (dMBD2/3) is highly derived, and no related protein has been identified in *C. elegans*. Human MBD4 is an atypical member of this family that functions in DNA repair rather than chromatin silencing.

down several preconceived ideas about the evolution of animal genomes. Rather than being simple, the common metazoan ancestor was genetically complex, containing many genes previously considered to be vertebrate innovations. In addition, *Acropora* and human sequences are often surprisingly similar. These data are a provocative reminder of the limited extent of our understanding of metazoan genome evolution and the potential hazards associated with extrapolating general evolutionary principles based on the model invertebrates. Whereas gene losses and modifications may obscure the picture in the model organisms and much of the animal kingdom remains to be explored, *Acropora millepora* provides a unique insight into the unexpectedly deep evolutionary origins of at least some vertebrate gene families.

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