Phylogenomic analysis of echinoderm class relationships supports Asterozoa

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While some aspects of the phylogeny of the five living echinoderm classes are clear, the position of the ophiuroids (brittlestars) relative to asteroids (starfish), echinoids (sea urchins) and holothurians (sea cucumbers) is controversial. Ophiuroids have a pluteus-type larva in common with echinoids giving some support to an ophiuroid/echinoid/holothurian clade named Cryptosyringida. Most molecular phylogenetic studies, however, support an ophiuroid/asteroid clade (Asterozoa) implying either convergent evolution of the pluteus or reversals to an auricularia-type larva in asteroids and holothurians. A recent study of 10 genes from four of the five echinoderm classes used ‘phylogenetic signal dissection’ to separate alignment positions into subsets of (i) sub-optimal, heterogeneously evolving sites (invariant plus rapidly changing) and (ii) the remaining optimal, homogeneously evolving sites. Along with most previous molecular phylogenetic studies, their set of heterogeneous sites, expected to be more prone to systematic error, support Asterozoa. The homogeneous sites, in contrast, support an ophiuroid/echinoid grouping, consistent with the cryptosyringid clade, leading them to posit homology of the ophiopluteus and echinopluteus. Our new dataset comprises 219 genes from all echinoderm classes; analyses using probabilistic Bayesian phylogenetic methods strongly support Asterozoa. The most reliable, slowly evolving quartile of genes also gives highest support for Asterozoa; this support diminishes in second and third quartiles and the fastest changing quartile places the ophiuroids close to the root. Using phylogenetic signal dissection, we find heterogeneous sites support an unlikely grouping of Ophiuroidea + Holothuria while homogeneous sites again strongly support Asterozoa. Our large and taxonomically complete dataset finds no support for the cryptosyringid hypothesis; in showing strong support for the Asterozoa, our preferred topology leaves the question of homology of pluteus larvae open.

1. Introduction

Echinoderms are composed of five extant classes, sea urchins (echinoids), starfish (asteroids), sea cucumbers (holothurians), brittlestars (ophiuroids) and sea lilies (crinoids). Although the modern classes appear in a relatively short time interval early in the fossil record (525–480 Ma) [1], extant crown group species in each class have more recent origins: present-day crinoids and echinoids radiated in large part after the Permian/Triassic extinction event for instance
The first molecular analyses using 18S rRNA genes found monophyletic echinoderms, but a questionable internal topology [8]; subsequent reanalysis of these data using more modern phylogenetic methods supports cryptosyringids [9]. Using 28S and 18S rRNA genes, Mallatt & Winchell [10] found strong support for Asterozoa. An analysis of 13 protein-coding sequences encoded in mitochondrial genomes [11] identified three lineages within the echinoderms—crinoids, ophiuroids and a clade consisting of asteroids, echinoids, holothurians). Inspection of this tree shows that the ophiuroids are particularly rapidly evolving, raising the likelihood that their position as sister to other eleutherozoan classes is the result of a long-branch attraction (LBA) artefact [12]. Janies and co-workers analysed combined molecular (seven loci) and morphological datasets from a large set of echinoderms. They produced trees supporting asterozoans and cryptosyringids (as well as other scenarios) and concluded that the overall phylogeny was sensitive to the choice of analytical methods [13].

Most recently, Pisani and co-workers presented an analysis of seven nuclear protein-coding genes and three nuclear ribosomal RNAs from four of the five echinoderm classes (crinoids, ophiuroids, asteroids and echinoids). Analysis of their full dataset supported the Asterozoa hypothesis (ophiuroids, asteroids) [14]. By careful removal of subsets of data expected to be problematic, they produced, in contrast, a tree supporting the cryptosyringid (ophiuroid, echinoid) clade. Their analysis did not include any holothurian sequences. There is the possibility that this omission might have resulted in an unnecessarily long echinoid branch rendering the dataset more susceptible to branch positioning artefacts. We have re-examined the question of echinoderm class relationships with a large dataset (219 nuclear protein-coding genes) including representatives from all five extant classes of echinoderms including data from two ophiuroids, two asteroids, two holothurians, four echinoids and a crinoid as well as data from three hemichordates and a cephalochordate as outgroup taxa.

2. Material and methods

Our analysis combined sequences from novel data sources and pre-existing protein and expressed sequence transcript databases, as outlined below.

(a) Sources of data

*Amphipholis squamata* were collected by Peterson mud grabs at a depth of approximately 40 m close to the Sven Loven Centre for Marine Sciences, Kristineberg, on the Gulmar fjord, Sweden (58°15′ N, 11°25′ E). A normalized cDNA library was constructed from mRNA of arm tissues from *A. filiformis* (regenerated and intact tissues) and sequenced using Roche 454 GS-FLX Titanium technology.

Adult *Amphipholis squamata* were collected in Argylle Creek on San Juan Island, Washington, and maintained in flow-through aquaria at Friday Harbor Laboratories. Brooded juveniles
at a range of stages of adult body plan formation were dissected out of the bursal sacs of the adult animals and flash frozen in liquid nitrogen. Following total RNA extraction, cDNA library construction was carried out by Express Genomics, Maryland, and sequencing of excised inserts was carried out using Roche 454 GS-FLX technology.

**Solaster stimpsoni** adults were collected by SCUBA at Eagle Cove San Juan Island, Washington, in April during their reproductive season. Adults were spawned by injection of 1-methyl adenine as described [15]. The positively buoyant embryos were cultured in running seawater tanks as described [16] with Sanger EST sequencing as described in [17,18].

Adult *Florometra serratissima* were collected by SCUBA at Bamfield Marine Station Sciences Centre, British Columbia, Canada. Mature oocytes were dissected from the adult female pinnules using forceps and fertilized with sperm obtained by rupturing testes. Embryos were cultured in glass bowls. Gastrula, early doliolaria and late doliolaria developmental stages were frozen in liquid nitrogen and total RNA prepared using the Ambion RNA aqueous mini kit. RNA samples were pooled and prepared for sequencing using the Illumina TruSeq stranded mRNA sample prep with oligo-dT selection then sequenced using HiSeq 101 bp paired-end reads.

**Eucidaris tribuloides** transcriptome sequences were obtained from the NCBI est_others database.

All other echinoderm nucleotide sequences were taken from the NCBI est_others database.

**(b) Preprocessing of nucleotide sequences**

High coverage and short read lengths mean that sequences produced using so-called next generation (i.e. Illumina, 454) technologies benefit from being preassembled using dedicated software.

We assembled a dataset of 46 312 223 2 × 101 bp paired-end sequences from the crinoid *F. serratissima* using the Inchworm program from the Trinity package [19]. This resulted in a set of 1 681 253 sequences.

**Amphiura filiformis** reads were assembled in MIRA v. 3.2.0rc3 [20], giving 35 742 sequences. *Amphipholis squamata* and *E. tribuloides* reads were assembled using the Newbler assembler, to give 53 028 and 21 969 contigs, respectively [21].

**(c) Orthologue identification and alignment construction**

We used a set of 12 625 *Saccoglossus kowalevskii* (Skow) sequences retrieved from the NCBI protein sequence database to search the predicted proteomes of the chordate *Branchiostoma floridiae* (Bflo), retrieved from the JGI and the sea urchin *S. purpuratus* (Spur) retrieved from the NCBI. We identified trios of sequences that formed consistent reciprocal best matches in each other’s proteomes and were therefore likely orthologues. Best matches were identified using blastp [22].

For all *Saccoglossus* sequences that were members of an orthologous trio (Skow, Bflo and Spur), we used tblastn to search a database of all echinoderm and hemichordate expressed sequences from the NCBI est_others database, with the addition of the *Florometra*, *Amphipholis*, *Amphiura*, *Solaster* and *Eucidaris* processed datasets described above and *Ptychodera flaev* sequences taken directly from the NCBI trace archive without preprocessing.

Significant hits to each *Saccoglossus* protein were extracted from the echinoderm nucleotide sequence databases and placed into species-specific groups. The nucleotide sequences in each species group were then assembled in isolation using CAP3 [23]. Assembled sequences thus produced were searched against metazoan protein sequences from the NCBI NR protein sequence database (blastp) and translated against the best match using the est_others program from the genewise package [24].

Each of the resulting assembled protein sequences was then searched against the combined proteomes of *Saccoglossus*, *Branchiostoma* and *Strongylocentrotus* using blastp, and only those for which the best hits were consistent with the members of the initial Skow, Bflo and Spur orthologous trio were retained.

Sets of proteins for each Skow, Bflo and Spur trio were then aligned with all predicted orthologous echinoderm and hemichordate proteins using PROBCONS [25]. A phylogeny was constructed for each protein alignment using ProML [26], using the WAG model [27], estimating the proportion of invariable sites, estimating the gamma distribution parameter allowing four gamma rate categories (WAG + I4) and optimizing the tree topology and branch lengths. Each phylogeny was then rooted with the *Branchiostoma* sequence (the chordate outgroup). Only those alignments that then yielded a monophyletic cluster of echinoderms were retained (i.e. no echinoderm sequences were allowed to branch within the hemichordates or between hemichordates and the root). This test will have the effect of excluding gene sets that include paralogous echinoderm sequences. Paralogous sequences would cluster outside a set of orthologous genes in a correct phylogeny. Rooting with the *Branchiostoma* orthologue will cause any paralogous echinoderm sequences to branch between this root and hemichordate (the presence of which is inevitable given the composition of our seed orthologue trios). Branching order of echinoderm classes within the echinoderm monophyletic group was not constrained during this process. Tree re-rooting and tests for monophyletic groups were performed using custom perl and python scripts. Final alignments were also screened for RNA contamination against all RNA sequences from the Rfam database [28], using tblastn [22].

**(d) Additional filters on final alignment**

Only proteins for which at least four out of five echinoderm classes were represented were included in the concatenated alignment (echinoids were invariably present owing to the presence of a *S. purpuratus* protein in the seed orthologue trios). We finally processed the alignment by visual inspection to remove unreliably aligned sections. The final concatenated alignment contained 219 genes.

**(e) Phylogenetic analyses**

*PhylProB* analyses were performed with the computationally intensive site-heterogeneous CAT + GTR + Γ mixture model taking approximately one month per analysis. This model accounts for across-site heterogeneities in the amino acid replacement process and is implemented in an MCMC framework by the program *PhylProB* v. 3.3d [29]. Two independent runs were performed with a total length of 6000 cycles, saved every 10 cycles. To construct the tree, the first 2000 points were discarded as burn-in, and the topology and posterior consensus support was computed on the 4000 remaining trees. We also checked for convergence of the two independent runs following burn-in of 2000 using bcpomp, which in each case showed a ‘maxdiff’ (maximum discrepancy) below 0.1.

In order to assess the reliability of trees, we calculated phylogenies on 50 bootstrap replicates (i.e. sampling with replacement to create an alignment the same size as the original) and 100 jackknife replicates (i.e. sampling without replacement, alignments 25% original length), in both cases using *PhylProB* with the CAT + GTR + Γ model. Chains were run for 1000 cycles, and consensus trees calculated using bcpomp, with 200 cycles discarded as burn-in.
datasets was analysed separately and the results compared. Genes ranging from the slowest to the fastest. Each of these four was divided into four approximately equal-sized quartiles containing order of their evolutionary rates and the resulting dataset of the rate of evolution for each gene. Genes were concatenated in was divided by the number of taxa on the tree to give an estimate tree (in estimated substitutions per position across all branches) was calculated using PHYML [26] and the total length of that tree (in estimated substitutions per position across all branches) was divided by the number of taxa on the tree to give an estimate of the rate of evolution for each gene. Genes were concatenated in order of their evolutionary rates and the resulting dataset divided into four approximately equal-sized quartiles containing genes ranging from the slowest to the fastest. Each of these four datasets was analysed separately and the results compared.

Recapitulating the approach of Pisani et al. [14], the number of changes at each site was calculated over a maximum-parsimony tree constructed with unresolved relationships between echinoderm classes. The number of changes for each character over the tree was normalized to correct for positions with missing data, by dividing by the number of changes by the number of taxa with a character at that position. The positions in the alignment were then divided into one sub-alignment containing heterogeneously evolving sites (comprised all invariant positions plus the most frequently substituting quarter of the variable sites) and a second sub-alignment containing the more homogeneously evolving positions comprised the remaining 75% of the more slowly evolving variable sites. These two datasets were analysed separately and the results compared.

Using an alignment for each gene, a maximum-likelihood tree was calculated using PhyML [26] and the total length of that tree was calculated using PHYML [26] and the total length of that tree (in estimated substitutions per position across all branches). We ran two runs of four chains each for a total of 500 000 generations well beyond convergence of the two runs. We also use individually best fitting empirical model as before. Ten replicates were run: 9/10 of the positions randomly drawn from the alignment were used as the learning set and the remaining 1/10 as the test set. For each model, MCMC were run for 1100 cycles, 100 being discarded as burn-in. Analysis using PHYLOBAYES [29] on the full dataset of 45 818 reliably aligned amino acids from 15 taxa produced the tree shown in figure 2. As widely recognized, the crinoids diverge first and form a sistergroup to the four other classes—the Echinozoa. Within the Echinozoa, the Asterozoa clade (asteroids + ophiuroids) and the Echinozoa (echinoids + holothurians) are recovered as sister clades. Within the echinoids, the cidaroid E. tribuloides is the deepest branching and within the echinoderms, the pycnoderids clustered together, as expected. All clades have a posterior probability of 1. This topology is supported by analyses using both the widely employed site-homogeneous WAG + G and GTR + G models (gamma with five rate categories) and the more complex site-heterogeneous CAT + GTR model (with the default Dirichlet process for estimating rates across sites [32]). We verified by cross-validation that the CAT + GTR + G model had a better fit to our dataset than the GTR + G (ΔlnL 228.36 ± 23.5159) and that the GTR + G had a better fit than WAG + G (ΔlnL 290.48 ± 17.613).

We performed 50 bootstrap replicates using the CAT + GTR + G model: 90% of replicates supported Asterozoa. Of the remainder, 8% placed ophiuroids as sister to holothurians, with only 2% supporting Cryptosyringida. We also performed 100 jack-kernel replicates using just 25% of the full dataset: the results with such a reduced dataset were naturally less robust; nevertheless 57% of trees support the asterozoan topology. As with the bootstrap samples, the next most common result in the jack-kernel set (25%) contained the unlikely grouping of holothurians and ophiuroids. Cryptosyringids sensu stricte (H,E,O) were observed in only 8% of trees, with a further 5% containing monophyletic cryptosyringids although with ophiuroids and holothurians as sister groups (H,O,E). In summary, both bootstrap and jack-kernel replication provide support for asterozoans. The cryptosyringids were the third best supported topology with both approaches, and less well supported than a grouping of holothurians and ophiuroids, which, to the best of our knowledge, has no serious support from other sources and can likely be discounted as a long-branch artefact.

The Asterozoa–Echinozaa topology has been supported by the majority of molecular analyses of this problem. By contrast,
the recent work by Pisani et al. addressing the possibility of systematic error due to unequal rates of evolution and saturation using an approach they call phylogenetic signal dissection supports, instead, the cryptosyringid clade (Echinozoa + Ophiuroidea). This result was supported in the Pisani et al. analysis only when using the portion of the alignment remaining after the constant and the most variable sites had been removed. We set out to replicate this approach with our larger and more taxonomically complete dataset. We used two approaches to test the effects of removing faster evolving and less reliable data: first, the site-based method as described, which we term site rate ranking (SRR); second, we classified each gene in our alignment into one of four quartiles according to its rate of evolution and analysed each of these quartiles in turn—we call this gene rate ranking (GRR). The GRR method has the advantage that it does not depend on pre-specifying any topology, whereas the SRR approach has the advantage that it treats each site independently.

Using either of these approaches with our dataset, we find no support for the cryptosyringid clade. Electronic supplementary material, figure S2, shows the results using the site-based SRR method. With the supposedly optimal portion of the alignment remaining after the constant and the most variable sites had been removed, we still find strong support for the Asterozoa–Echinozoa topology (electronic supplementary material, figure S2a). The support is slightly lower than when using the whole alignment (support for Asterozoa pp = 0.94), presumably because the alignment is shorter (16678 sites compared to 46913 for the complete alignment) and hence less informative. Using the suboptimal sites (constant and variable), we recover an improbable topology with Holothuroidea and Ophiuroidea as sister taxa (both are long branches) and the relative positions of echinoids, asteroids and the holothurian/ophiuroid group are unresolved (electronic supplementary material, figure S2b).

Earlier support for cryptosyringids came from a dataset that did not include holothurians [14] and so we repeated the SRR method with this class excluded. We recovered identical relative positions for each of the remaining classes and so this difference in taxonomic representation does not explain the difference between our results and those of Pisani et al. [14] (results not shown).

Figure 3 shows the results from the gene-based GRR approach. The slowest quartile (most reliable) shows the strongest support for Asterozoa (pp = 1, figure 3a). The second and third quartiles still support this clade but with diminishing support (second quartile pp = 0.83, figure 3b; third quartile pp = 0.71, figure 3c). The fourth quartile (fastest evolving genes and likely least reliable) places the ophiuroids as sister group to the Echinozoa + Asteroidea, presumably due to LBA between the relatively long-branched ophiuroids and the outgroups (figure 3d). All other aspects of the tree (including the Echinozoa clade) do not change as the datasets get faster and all other clades receive the maximum support (pp = 1) in analyses of all four quartets. The difference in rate is very evident when comparing these trees (shortest (figure 3a) to longest (figure 3d)), which are all drawn to the same scale.

We conducted two experiments using analyses performed with MrBayes in which we partitioned the dataset by genes
and allowed genes to adopt firstly gene-specific empirical models of amino acid substitution and secondly both gene-specific empirical models and a gene-specific rate. In both experiments, the topology was the same as in figure 2 with posterior probability of 1.0 for all nodes.

4. Discussion

(a) Echinoderm phylogeny

All of our analyses unequivocally support a clade of Ophiuroidea plus Asteroidea: Asterozoa. Despite using two methods to stratify our data by evolutionary rate, we found no support for the alternative Cryptosyringida hypothesis of a sister group relationship of Ophiuroidea and Echinoidea — Holothuroidea. Our dataset is considerably larger than others that have been brought to bear on this question, includes at least two members of each of the relevant classes and deep sampling of the closest outgroup (the crinoid). A priori, we might expect such a dataset to produce a more reliable result, and we have not been able to identify confounding factors. Our trees do reveal very short branches leading to the asterozoan and echinozoan clades, however. These divergences appear to have taken place in a compressed time interval, a fact perhaps underscored by the absence of any miRNAs that may have helped resolve the relative positioning of these taxa [14]. Further molecular testing of our topology might come from even deeper taxon sampling or new types of data. The order of the echinoid Hox genes, for instance, at least as observed in S. purpuratus, is derived relative to that seen in hemichordates, and apparently coupled with this, the HOX4 orthologue has been lost [33,34]. HOX4 orthologues have, however, been identified in crinoids [35] and asteroids [36]. Their status in ophiuroids is uncertain (the Stegophiura sladeni sequence referred to as SS-11 in the previous study [37] is not convincing), but our phylogeny would be problematic if ophiuroids and echinoids shared a complex molecular character state [38], such as a Hox cluster rearrangement and gene loss, to the exclusion of asteroids. We are not aware of any other complex molecular character states that might be informative for this phylogenetic question: the systematic exploration of their existence, presence and absence must await complete genome sequences of representatives of other echinoderm classes.

(b) Asterozoa and the origins of the larval skeleton

The most recent molecular study to address echinoderm phylogeny [14] stated that their result (i.e. supporting cryptosyringids) ‘confirms that that the morphologically similar pluteus larval stages of echinoids and ophiuroids are indeed homologous rather than convergent’. It is not clear, however, that the larval skeleton can be used as a phylogenetic character to distinguish between the alternative phylogenetic trees without making assumptions about the relative ease of gain or loss events (electronic supplementary material, figure S3). Under
the scenario of a single origin for the larval skeletons (the evidence for which we review briefly below), the cryptozygzing hypothesis is indeed more parsimonious than the asterozoan, requiring a single loss of the skeleton in holothurians (and concomitant reversal to a auricularia/bipinnaria type of larva), with the asterozoan hypothesis requiring an additional loss/reversal in asteroids (electronic supplementary material, figures S3a and S3c). If a dual origin of the larval skeleton is favoured, the two phylogenetic scenarios are indistinguishable on parsimony grounds (so far as it relates to the skeleton) (electronic supplementary material, figures S3b and S3d).

Although the larval skeleton seems intuitively to be a complex trait, in the sense that it might be regarded as unlikely to have evolved twice, all echinoderm classes share an adult skeleton, and the evolution of the larval skeleton may be the result of a relatively simple redployment of this, making the possibility of convergence more likely. Gao & Davidson [39], comparing larval and adult skeletogenesis, have identified the genes specifically required for embryonic activation of the skeletogenesis network in the echinoderm *S. purpuratus*, showing that this is only a small component of the overall skeletogenetic GRN. It is worth noting that the key gene in the embryonic skeletogenetic GRN, Pmar1, involved in the establishment of the double negative gate that de-represses skeletogenesis [5] currently appears to be an echinoderm autapomorphy. We could not find evidence of Pmar1 orthologue presence in any taxa in our echinoderm EST database (or non-echinoderm databases) except for *S. purpuratus*. Although Vaughn et al. [40] mention an unpublished ophiuroid Pmar1 orthologue, their inability to find Pmar1 transcripts expressed in any stage of brittlestar development supported their conclusion that ‘embryonic skeleton formation in sea urchins and brittle stars represents convergent evolution by independent co-optation of a shared pathway used in adult skeleton formation’ [40, p. 14].

The suggestion that the echinoid larval skeleton may be a derived state is further supported by McCauley et al.’s [4] analysis of holothurian embryogenesis. Based on an analysis of the embryonic expression patterns of mesodermal regulatory genes in *Parastichopus parvimensis*, they suggest ‘that the ancestor of the sea cucumber + sea urchin clade may have had a mesoderm with a regulatory state similar to that of extant sea cucumbers and may have developed only a rudimentary larval skeleton’ [41, p. 5], and argue that, as such, holothurians are unlikely to have lost a complex larval skeleton. If we accept the sister group relationship of echinoderms and holothurians, this in turn suggests that the larval skeleton was not a plesiomorphy of these two taxa and thus unlikely to be a plesiomorphy of any possible clade including the ophiuroids. Other authors have argued on morphological grounds that the two kinds of plutei are convergent forms [42,43], but as the defining feature of the pluteus is its skeleton, most desireable would be a comparison of the fine details of the developmental genetics responsible for initiating production of the larval skeletons, which we would expect to more exactly overlap in homologous plutei. We emphasize that such evidence would be useful for ruling out the scenario of a single origin for the pluteus within cryptozygzing (electronic supplementary material, figure S3c), not for distinguishing between the dual origin scenarios—however, our molecular evidence clearly favours the Asterozoa (i.e. electronic supplementary material, figure S3b).

(c) Adult morphology

The cryptozygzing ovoids their name to the fact that their radial water vascular system and radial nerve become covered during development, a trait that was viewed as a synapomorphy of the group [44]. In later work, however, Smith and co-workers [7] pointed out that the developmental mechanism by which this occurs differs within the echinoids, suggesting that its presence in some instances may be due to convergence, and as such, it would not be a reliable phylogenetic marker. To the best of our knowledge, there has been no work on the molecular mechanisms leading to covered radial nerves and water vascular system in ophiuroids and echinoids. Under the Asterozoa hypothesis, we would predict differing molecular mechanisms, suggesting parallel evolution of the trait in echinoids and ophiuroids, unless the trait were secondarily absent from asteroids, in which case the fossil record may be the best source of evidence.

If our inference of monophyletic Asterozoa is correct, it means that the hypothesis of a star-like common ancestor of Eleutherozoa can neither be rejected nor supported. This in turn has implications for the interpretation of the fossil record: in particular, based on our analysis, there is no reason to rule out a non-stellate ancestor for the echinoid–holothurian stem group.

Acknowledgements: We thank Sam Dupont (University of Gothenburg, Kristineberg) and David Dylus (UCL) for helpful comments.

Funding statement. C.J.L. acknowledges funding from: NASA Exobiology 1-EXO11-0135. C.B.C. acknowledges funding from the NSERC discovery grants programme. M.J.T. is supported by a Royal Society Wolfson Research Merit Award.

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