



## Resolving phylogenetic signal from noise when divergence is rapid: A new look at the old problem of echinoderm class relationships

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### ABSTRACT

Resolving evolutionary relationships in groups that underwent fast radiation in deep time is a problem for molecular phylogeny, as the scant phylogenetic signal that characterises short internal branches is generally swamped by more recent substitutions. We implement an approach, that maps how the support for rival phylogenies changes when analysing subsets of sites with either faster and more heterogeneous rates or slower and more homogeneous rates, to address a long-standing problem in deuterostome phylogeny – the interrelationships of the eleutherozoan echinoderm classes. We show that miRNA genes are phylogenetically uninformative as to the relationships of asteroids, echinoids and ophiuroids, consistent with a rapid radiation of these groups as suggested by their fossil record. Using three nuclear rRNAs and seven nuclear housekeeping genes, we map the support for the three possible phylogenetic arrangements of asteroids, ophiuroids and echinoids when moving between subsets of the data with very similar or very different rates of evolution. Only one of the three possible topologies (asteroids (ophiuroids + echinoids)) strengthens when the most rate-homogeneous subset of data are analysed. The other two possible pairings become stronger in a less reliable data subset, which includes the fastest and thus homoplasy-rich data in our alignment. Thus, while superficial analysis of our concatenated alignment identifies asteroids and ophiuroids as sister taxa, more thorough analyses suggest that ophiuroids may be more closely related to echinoids. Divergence of these echinoderm groups, using a relaxed molecular clock, is estimated to have occurred within ~5 million years. Our results illustrate that the analytic approach of phylogenetic signal dissection can be a powerful tool to investigate rapid radiations in deep geologic time.

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### 1. Introduction

Both morphological and molecular approaches to phylogenetic reconstruction work well when divergences between taxa are separated by relatively long time intervals, as the accumulation of substantial numbers of derived characters in the stem lineages creates a strong phylogenetic signal. However, when divergence occurred rapidly in deep time and stem lineages are of short duration, accurate phylogenetic reconstruction is difficult. This is because continuing evolution results in convergence and reversals that ultimately overwhelm the weak signal in short internal branches. In such situations, unequal rates of evolution can lead some branches to accumulate a significantly larger number of substitutions leading to the well-known problem of long-branch attraction (LBA: Felsenstein, 1978). While LBA has long been recognised as a problem, how best to identify trees affected by LBA and tease out historical signal from systematic biases remains a major

challenge (Brinkmann and Philippe, 1999; Ruitz-Trillo et al., 1999; Pisani, 2004; Lartillot and Philippe, 2008; Jeffroy et al., 2006; Sperling et al., 2009; Rota-Stabelli et al., 2010). Indeed, while the signature of rapid divergence is a phylogenetic tree where branching order cannot be resolved with confidence, LBA can confuse the picture causing the recovery of artefactual groups with very high support (Jeffroy et al., 2006).

One problematic area of the metazoan tree concerns how the five echinoderm classes are related (Smith et al., 2004; Janies et al., 2011). Both morphology and molecular data place crinoids as sister group to the other classes (echinoids, asteroids, ophiuroids, holothurians), and pair echinoids and holothurians together. Yet the interrelationships of asteroids, ophiuroids and the echinoid–holothurian clade remain disputed. Morphological data favours either an asteroid–ophiuroid pairing (Mooi and David, 2000) or an ophiuroid plus echinoid–holothurian pairing (Littlewood et al., 1997), whereas different molecular analyses have found support for all three possible groupings (Field et al., 1988; Littlewood et al., 1997; Janies, 2001; Mallatt and Winchell, 2007; Pereske et al., 2010; Janies et al., 2011; Letsch and Kjer, 2011).

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These echinoderm clades pose a particularly acute problem for molecular phylogenetic analyses because they underwent crown group diversification long after they had split from one another and all three have long stem groups that cannot be broken up by selective sampling of the modern fauna, making them particularly susceptible to LBA.

## 2. Materials and methods

### 2.1. Molecular data assembled

Total RNA was collected from the ophiuroid *Ophiopholis* and a small RNA library constructed and sequenced following Wheeler et al. (2009), resulting in 3804 parsed non-redundant reads. These were then compared with previously published small RNA libraries drawn from an asteroid (*Henricia sanguinolenta*), echinoid (*Strongylocentrotus purpuratus*), hemichordate (*Saccoglossus kowalevskii*) and other metazoans published previously and analysed by miRMiner (Wheeler et al., 2009) for known and potentially novel miRNAs (Table S1).

Six nuclear housekeeping genes (aldolase, methionine adenosyltransferase, ATP synthase beta chain, elongation factor 1 alpha, triosephosphate isomerase and phosphofructokinase) were sequenced from the ophiuroid *Ophiopholis* sp. following the protocol described in Sperling et al. (2009). These sequences have been deposited in Genbank under accession numbers (JN716365–JN716370). Sequences for *Aplysia californica*, *Alvinella pompejana* and *Tubifex tubifex*, as well as three genes for *Carinoma mutabilis*, were downloaded from the NCBI trace archives. Unpublished sequences from *Chaetopleura apiculata* and *Leptochiton asellus* were kindly provided by J. Vinther (Yale University). Sequences for other lophotrochozoan taxa were taken from previously published reports (Peterson et al., 2004), and new sequences were manually added to the pre-existing alignment used, for example in Sperling et al. (2011). Data for ribosomal 5.8S, 18S and 28S ribosomal genes for 22 deuterostome, 35 lophotrochozoan, and 15 ecdysozoan taxa were assembled, either taken directly from Mallatt et al. (2010) or downloaded from the NCBI Genbank website and manually aligned to the Mallatt et al. (2010) sequences. Chimaeras at the generic level were permitted when data for the same species were not available. After the removal of minor indels, the amino acid matrix was 88% complete and the ribosomal matrix was 76% complete. The seven nuclear housekeeping genes (2049 amino acids in total) and three ribosomal genes (4682 nucleotides in total) were concatenated for analysis.

### 2.2. Sequence analysis

#### 2.2.1. Conventional phylogenetic analysis

The protein and rRNA partitions were first independently analysed to investigate the nature of the principal signal (Pisani and Wilkinson, 2002) in these data sets. Protein analyses were performed using the heterogeneous CAT-GTR model, and rDNA analyses were performed using the GTR + G model, which proved to be the best fitting model (selected using MrModeltest) for our nucleotide data. CAT-GTR analyses were performed in Phylobayes V. 3 (Lartillot and Philippe, 2004). We used posterior predictive analysis as implemented in Phylobayes (see also Sperling et al., 2009) to discover whether the taxa of interest (i.e. the echinoderms) were compositionally homogeneous or heterogeneous.

The rRNA and protein partitions were concatenated and analysed under mixed models using Bayesian and Maximum Likelihood (ML) analyses. Maximum Parsimony (MP) and Neighbour Joining (NJ) (with uncorrected P distances and no gamma correction) were also performed. Bayesian analyses were performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001), ML analyses

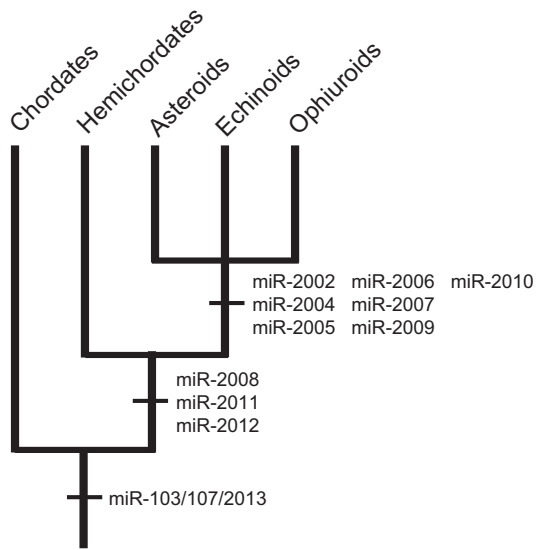
were performed using RAxML (Stamatakis, 2006), while MP and NJ analyses were performed using PAUP4b10 (Swofford, 2002). Support for nodes found in the MP, NJ and ML analyses was estimated using the bootstrap, with 500 replicates for MP and NJ (but see Supplementary information) and 5000 replicates for ML.

For all Bayesian mixed models analyses both the rRNA and protein partitions were modelled using GTR + G. Sperling et al. (2009) showed that for this protein data set, GTR + G is the best fitting amongst the homogeneous substitution models implemented in MrBayes, whilst we showed here that GTR + G is the best fitting model for our nucleotides partition. CAT-GTR analyses could not be performed for the concatenated data set because of software limitation (Lartillot, pers. comm.). For the ML analyses the protein partition was modelled using LG + G. The nucleotide partition was modelled using GTR + G.

#### 2.2.2. Phylogenetic signal dissection

Both the rRNA and the Protein data sets were partitioned into sets of “homogeneously evolving” and “heterogeneously evolving” sites using a modification of Brinkmann and Philippe’s (1999) slow-fast approach (see Sperling et al., 2011 for justifications). This method assigns rates to characters semi-independent of tree topology. The characters in the rRNA and protein data sets were independently ranked according to their evolutionary rate (estimated as slow-fast parsimony scores) and partitioned into four quartiles. For each data set (proteins and rRNAs) characters were split into two groups: the first containing all the sites in the fourth quartile plus invariant sites, the second contained all the variant sites in the first, second and third quartiles. The characters in the first data partition represent a combination of sites with highly heterogeneous rates (i.e. very fast and constant sites only). This partition included 1247 AA and 3206 NN positions, of which 748 AA and 2332 NN positions were constant and 499 AA and 874 NN where deemed to be fast evolving. Because of the extreme rate variation (including constant and fast evolving sites only), and the high substitution rates and homoplasy levels of the variable characters it includes, this data partition presents a hard phylogenetic problem, and is prone to generate phylogenetic artefacts (e.g. LBA) even when analysed using well-fitting, parameter-rich models. The second data partition is composed of phylogenetically more reliable, rate-homogeneous, characters of slow to intermediate evolutionary rate. This partition includes 811 AA and 1476 NN (all of which are parsimony informative) and is more likely to support relationships that represent historical signal (see Sperling et al., 2009, 2011; Rota-Stabellini et al., 2010).

We then evaluated the strength of the signals supporting the three possible arrangements of asteroids, echinoids and ophiuroids residing in the three data sets (i.e. all sites, rate-heterogeneous sites and rate-homogeneous sites), under three, differently performing, methods – Parsimony, Neighbour Joining and Bayesian analysis. The fit to data of the three topologies (see Fig. 1) into which asteroids, echinoids and ophiuroids can be arranged were compared using Bayes Factors (BF; e.g., Sperling et al., 2010; Holton and Pisani, 2010) as follows. For each data set (homogeneous, heterogeneous and all sites), and each sister-group hypothesis (E + O, A + E and E + A), a constrained tree search (of 2 runs and four chains per run) was performed in MrBayes (Huelsenbeck and Ronquist, 2001). Each constrained tree search was run for 5,000,000 generations and a burn-in of 2,500,000 generations was used. This burn-in period was sufficiently long to allow each analysis to converge, and generated an identical number of data points (per data set and hypothesis) to calculate the BF. For each data set, the MrBayes “.p” file corresponding to the chain of maximal marginal likelihood across all trees (estimated using the harmonic mean) was selected, and used to estimate the BF for each pair of considered hypotheses in Tracer v1.5.1 (Rambaut and



**Fig. 1.** MicroRNA phylogeny of deuterostomes highlighting the lack of phylogenetically informative characters for resolving echinoderm class relationships.

Drummond, 2007). Because the variance around the BF harmonic means can be extremely large (Lartillot and Philippe, 2005), we followed the suggestion of Marc Suschard (unpublished but see [http://groups.google.com/group/beast-users/browse\\_thread/thread/3e9d7da1eeb9d6c8/9e3aa8eb29c76978?pli=1](http://groups.google.com/group/beast-users/browse_thread/thread/3e9d7da1eeb9d6c8/9e3aa8eb29c76978?pli=1)), that BF be calculated multiple times from the same data to estimate how much the results vary. Here, we have calculated the BF twice, starting from two independent MrBayes runs (time and computational limitations prevented us from performing more independent tests). In addition, all our BF results are presented in association with Standard Errors around the calculated harmonic means. For all BF analyses the protein and the nucleotide partitions were modelled using two unlinked GTR + G models.

To estimate the strengths of alternative signals in the three data sets we performed bootstrap analyses under MP, NJ and ML and compared the support for the three alternative topologies. Similar analyses could not explicitly be performed in a Bayesian framework, because, for each of the tree data sets, only one of the alternative hypotheses was supported. For each data set, some of the alternative hypotheses received low to extremely low levels of support, indicating that the strength for that signal in that data set was minimal. To evaluate whether these low support values represented an artefact of the bootstrap resampling, or a real feature of the data, for each data set we performed multiple bootstrap analyses (only under MP and NJ). In these analyses the number of replicates was incrementally augmented. Bootstrap analyses were performed (in Paup4b10) using 100, 500, 1000, 5000 and 10,000 replicates by which time the signal had stabilised around a given value.

### 2.3. Molecular clock analyses

All relaxed molecular clock analyses were performed using the software Phylobayes version 3 (Lartillot and Philippe, 2004) following the protocol of Sperling et al. (2010) and using the CIR model, an autocorrelated model that fits this data set better than uncorrelated models (Sperling et al., 2010). An additional 29 sponge, seven cnidarian and two non-metazoan outgroups (the choanoflagellate *Monosiga brevicollis* and the yeast *Saccaromices cerevisiae*) were added to the dataset to maximise the number of calibration points. Clock analyses used a fixed topology based on the results of the homogeneous data set only (i.e. with an ophiu-

roids plus echinoids grouping), combined with the results of Sperling et al. (2010). Branch lengths for this fixed topology (Table S9) were re-estimated under the CAT-GTR model using only the protein alignment. A total of 24 calibration points, spread phylogenetically throughout Metazoa and spaced temporally from the Miocene to Cryogenian, were used (Supplementary data, Table S2). Using Phylobayes two chains were initially run using soft bounds and allowing 5% of the prior probability density to lie outside of the minimum–maximum interval defined for each calibration point. Further analyses were performed to test the effect of different levels of relaxation on the recovered ages. We calculated divergence times allowing 10%, 25% and 50% of the prior probability density of each calibration point to lie outside the min–max interval defined by the provided calibration points. Analyses were also run with no-data to test the effect of our calibrations on the unconstrained nodes; this was done to test whether “composite calibration points” (i.e. the effect of multiple surrounding calibration points on intervening nodes) could have biased our results. The root node in our molecular clock analyses represents the split between Fungi and the Holozoa, and all the above-mentioned analyses were run using a prior root age of 1000 Ma and a standard deviation of 100 Ma. Analyses performed using the 5% relaxation level were also performed using a significantly deeper prior root (1600 Ma) and a SD of 700 Ma to test the effect of the root-prior on our divergence times.

## 3. Results

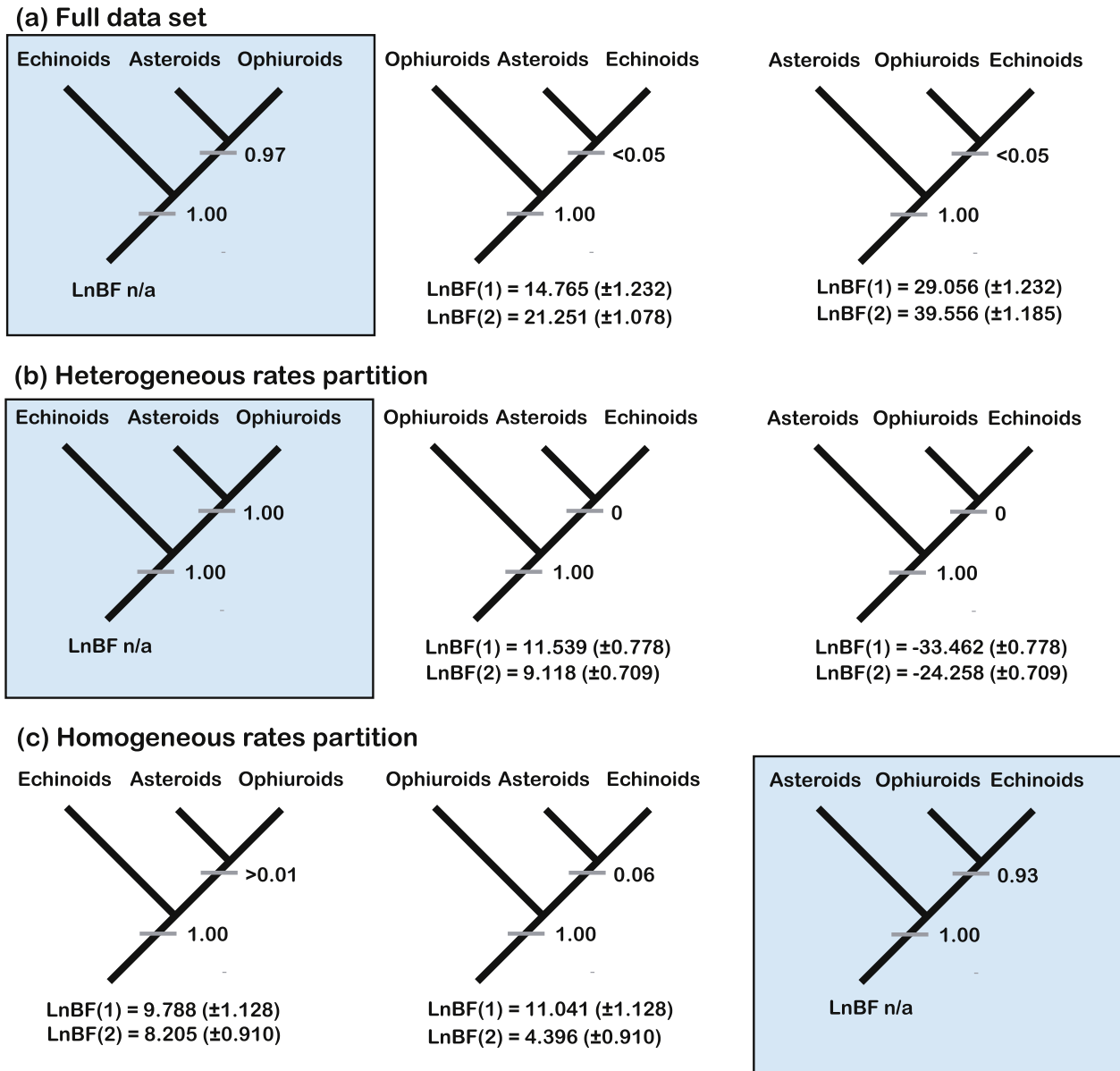
### 3.1. MicroRNA markers in echinoderms

Virtually all expected miRNAs were discovered in our ophiuroid small RNA library, including the deuterostome-specific miR-103/107/2013, the ambulacrarian-specific miRNAs miR-2008, -2011 and -2012, and seven echinoderm-specific miRNAs (Fig. 1). No miRNA shared between any two of the three echinoderms to the exclusion of the third was found (Supplementary data, Table S1). Only six potential miRNA sequences were shared among at least two of these three taxa, but none of these was a novel miRNA (three were transfer RNA sequences, and the other three were edits to known miRNAs).

### 3.2. Phylogenetic analyses of standard sequence data

Posterior predictive analysis showed that amino acid sequences in all species relevant to this study (and the great majority of the species in this data set) are compositionally homogeneous (Supplementary data, Table S3). The nucleotide data do, however, show compositional heterogeneity, although the ophiuroid and most echinoid sequences are compositionally homogeneous (Supplementary data, Table S4). This is not considered a problem for our analyses, as we never observe compositionally heterogeneous taxa grouping together.

As the relationships between outgroup organisms are essentially static across analyses, only the three taxa under consideration – echinoids (E), asterozoa (A) and ophiurozoa (O) – are discussed. Analyses of the rDNA (Supplementary Fig. S1) and of the protein data (Supplementary Fig. S2) partitions found low support for the grouping A + O (Posterior Probabilities (PP) = 0.56 (rDNA) and 0.49 (protein)). This group is then sister to the echinoids in both the rDNA (PP = 0.97) and protein data (PP = 1). Analyses of the concatenated (proteins and rRNA) data set performed under mixed models (Fig. 2A, left; Supplementary Fig. S3) also support a sister group of A + O, but with higher support (PP = 0.97; ML-BP = 0.71). This group is then the sister of the echinoids (PP = 1). Although data concatenation increased the



**Fig. 2.** Cladograms summarising levels of Bayesian Factors support for alternative potential topologies under different data partitions of the combined rDNA and protein sequences A, full sequence; B, partition of the quartile of fastest evolving sites plus invariant sites (heterogeneous partition); C, partition of the slow and intermediate evolving sites only (homogeneous partition). Shaded box indicates best-supported topology in each case.

support for A + O (Fig. 2A), a common feature of these trees (Supplementary Figs. S1–3) is that the asteroid and ophiuroid terminal branches are long whilst the internal branch uniting them is very much shorter, raising the possibility that the pairing of asteroids and ophiuroids could be the result of LBA.

### 3.3. Phylogenetic signal dissection of rate-homogeneous and rate-heterogeneous data partitions

The fit of our data sets to the three alternative phylogenetic hypotheses was tested using Bayes Factors (Supplementary data, Tables S5–7). Results obtained in the two independent BF analyses are in full agreement, and comparisons of the estimated harmonic means (with their bootstrapped confidence intervals) show that uncertainty around the estimated harmonic means should not be a problem for our analyses.

When all sites are used BF clearly supports the A + O pairing and finds least support for the E + O pairing (Fig. 2A). Analysis of the

heterogeneous data partition also identified strong support for the pairing A + O (PP = 1; ML-BP = 79 Fig. 2B). The homogeneous data partition, however, supports a different set of relationships, pairing O + E (PP = 0.93 Fig. 2C). Support for an A + O, or E + A pairing is minimal in this subset of data (less than 0.1 for both hypotheses; Fig. 2C). Exactly the same pattern is recovered when the analyses are repeated under ML, with higher support for an E + O pairing appearing in the homogeneous data partition, although support values are much lower and non-significant. Thus, the signal that groups A + O is strongest in the heterogeneous partition, whereas that for E + O is strongest in the homogeneous partition. Indeed, E + O is the only grouping that is better supported in the homogeneous partition (Fig. 2C), than in either the heterogeneous data partition or the full alignment. Note that the precise level of support for this group is method dependent, being higher under Bayesian analysis than maximum likelihood. This in part is to be expected as the bootstrap is known to be over-conservative whilst posterior probabilities might be too optimistic (e.g. Douady et al.,

2003). However, in this case the lower support obtained under ML (given also the substantial difference in support observed), most likely reflects the poor ability of MCMC methods to deal with complex models and mixed data sets (e.g. Lartillot and Philippe, 2004). Accordingly, we suggest the results of the Bayesian analysis, in this specific case, may better describe the strength of the signal in the compared data sets.

When the full data set is analysed using NJ and observed distances, a method and a distance measure that perform poorly and are easily swayed by LBA, support is again found for an A + O pairing (BP = 55%) (Fig. 3). The support for this group reaches a maximum of 65% in the NJ analysis of the heterogeneous data, and drops to 33% in the NJ analysis of the homogeneous data. Parsimony analysis (which is also easily swayed by LBA) of the complete data set finds virtually no support for E + O pairing (BP = 3%) and minimal support for the A + O group (BP = 15%), favouring instead a pairing of E + A (BP = 81%) (Fig. 3). As found with the NJ analyses, when the heterogeneous data are analysed with MP, the support for A + O rises to 36%, whereas support for E + A decreases to BP = 63% and support for E + O drops to zero. However, when the homogeneous data are analysed with MP, support for E + O increased to 55%, whilst support for E + A and A + O decreased to 26% and zero, respectively. That these differences are not stochastic variations associated with the heuristic nature of the bootstrap is demonstrated by the consistency of the differences observed (Fig. 3).

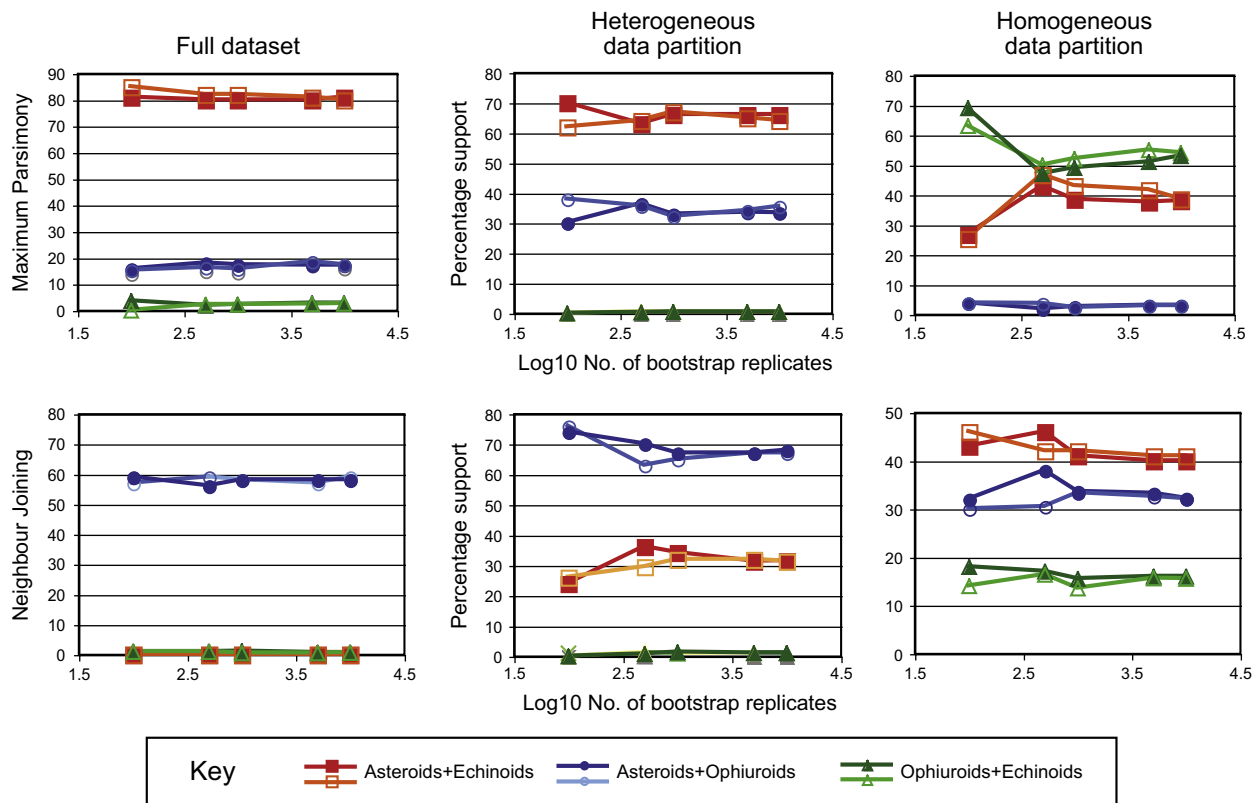
#### 3.4. Molecular divergence estimates

Using a relaxed molecular clock methodology we find that the divergence amongst the sampled eleutherozoan echinoderms is estimated to be Early Ordovician ~480 Ma (95%CI = 505–446)

(Fig. 4). Consistent with the fossil record (Dean-Shackleton, 2005; Smith and Savill, 2001), we estimate that ophiuroids and echinoids diverged very soon afterwards, roughly 475 Ma (95% CI = 501–440) (Fig. 3). Thus, this is indeed a very rapid divergence, spanning approximately 5 million years. Sensitivity analyses indicate that our dates are robust and unlikely to have been caused by the use of inappropriate fossil calibrations. Running the analyses under the priors shows that our set of calibrations seem appropriate to address the problem at hand (not shown). Relaxing the soft bounds to allow up to 10%, 25%, or 50% of the prior probability density to lie outside of the minimum–maximum interval of each considered calibration point caused negligible changes to estimated echinoderm ages, and in two cases (10% and 25%) cases still recovered divergence times that lay within the 95% confidence interval of the analysis run under the default 5% relaxation level (Supplementary data, Table S8). Finally, changing the root prior age did not significantly affect our recovered divergence times (Supplementary data, Table S8).

#### 4. Discussion

MicroRNAs are a diverse family of small, non-coding regulatory genes present throughout Bilateria. Because they are continually added to over time, rarely change in primary sequence and are only rarely secondarily lost in most taxa, they are considered reliable phylogenetic markers (Sperling et al., 2010; Sperling and Peterson, 2009; Heimberg et al., 2010; Rota-Stabellini et al., 2010). Yet unexpectedly we found no unique microRNAs to resolve the asteroid–echinoid–ophiuroid trichotomy (Fig. 1). Polytomies that cannot be resolved using microRNAs must be considered as potentially having undergone rapid divergence, a possibility also suggested



**Fig. 3.** Plots of bootstrap support for the asteroid + ophiuroid, asteroid + echinoid and ophiuroid + echinoid pairings in the rDNA and protein coding gene sequences as described in this paper. Bootstrap analyses were carried out using 100, 500, 1000, 5000 and 10,000 replicates under both maximum parsimony and neighbour joining for the full dataset, the heterogeneous data partition and the homogeneous data partition.

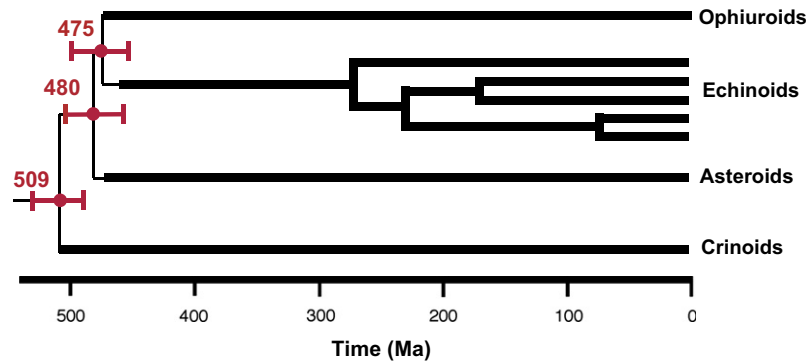


Fig. 4. Time of divergence estimates based on a relaxed molecular clock approach (see text). Thick lines = known fossil record.

by the fossil record (Smith, 1988), the volatile phylogenetic signal that emerges from gene sequence data (Fig. 2), and our molecular divergence estimates (Fig. 4).

Reconstructing relationships of clades that have radiated rapidly deep in geological time has proved to be particularly difficult, and the general approach adopted to address such problems has been to try and tease out a weak signal using larger and larger data sets (e.g. Holton and Pisani, 2010; Rota-Stabellini et al., 2010; Dunn et al., 2008; Hejnol et al., 2009). However, while increasing the dimension of the data set can eliminate stochastic errors, it will also exacerbate systematic errors like LBA (Sperling et al., 2009; Pick et al., 2010). Indeed, it is a misconception that simply adding more data will eventually lead to the recovery of the correct phylogeny; if the data are affected by LBA then the opposite will happen (Jeffroy et al., 2006).

The comparison of phylogenies obtained using differently fitting substitution models (and differently performing phylogenetic methods) has previously been used to identify LBA artefacts. This is because well-fitting substitution models (e.g. the CAT model of Lartillot and Philippe, 2008), and optimal outgroup selection (Rota-Stabellini and Telford, 2008) can help reduce LBA (Sperling et al., 2009; Rota-Stabellini et al., 2010; Rodriguez-Ezpeleta et al., 2007). An alternative, but less frequently used, strategy to circumvent LBA is to exclude sites with high evolutionary rate (i.e. site stripping) from the analyses (e.g. Brinkmann and Philippe, 1999; Rodriguez-Ezpeleta et al., 2007; Pisani, 2004; Sperling et al., 2009; Rota-Stabellini et al., 2010).

Wägele (1999) identified three classes of LBA artefact: sympleisyomorphy trap (Type I LBA), erosion of phylogenetic signal (Type II LBA), and misleading and invisible attraction due to non-homologous similarities (Type III LBA). Each affects tree topology in a different way, producing artefactual topologies with different characteristics. However, all stem from the same phenomenon: the existence of substantially different lineage-specific substitution rates. Site-stripping approaches that exclude sites that accumulate substitutions at high rate (thus contributing to LBA), certainly help circumvent Type II and III artefacts, but it is unclear how much site stripping can help circumventing Type I artefacts. However, the application of site stripping should not exacerbate Type I LBA artefact, so long as only fast evolving sites are excluded. This is because rapidly evolving sites tend to be saturated and hence rich in homoplasy (including reversals) and poor in true apomorphies. True apomorphies are concentrated rather in sites of either slow or (most likely) intermediate rate, which we retain. Accordingly, exclusion of fast sites should not increase the true plesiomorphy to true apomorphy ratio in the data set, which is ultimately responsible for Type I LBA artefacts (see Wägele, 1999). In any case, it is clear that, even for data affected by Type I LBA, if noisy (fast) sites are excluded true but weak phylogenetic signal is more likely to emerge. Hence, we would expect that

exclusion of sites of high rate (where multiple substitution are more likely to accumulate) should have a generally positive (or in the worst case neutral) effect independent of the LBA type affecting a data set.

It is important to bear in mind that site-stripping based methods are not the only possible approach to attempt circumventing systematic artefacts (see Jeffroy et al., 2006 and references therein), and they should not be considered a generalised panacea. Their utility is limited to deep time studies where anciently acquired substitutions at fast evolving sites are likely to have been erased by subsequent substitutional events.

In contrast to standard site stripping approaches, where only sets of slowly evolving sites are analysed, our approach (see also Sperling et al., 2009, 2011) compares the strength of phylogenetic signals in both the slow and fast evolving data, thus effectively associating the various signals to subsets of data. Signals associated with fast evolving sites most likely characterise artifactual groups, while those associated with slowly evolving sites are more likely to support real clades. Our results suggest that there is a partitioning of the signals within this data set, with the signal supporting the pairing of asteroids and ophiuroids concentrated in the fast (i.e., heterogeneous) positions and that supporting the pairing of echinoids and ophiuroids concentrated in the slow (i.e., homogeneous) positions. Support for the pairing of echinoids and asteroids is more widely distributed with some support for this group present in the homogeneous partition but with the majority residing in the heterogeneous partition.

These results clearly illustrate a serious, often underestimated potential pitfall of supermatrix analyses, that a clade with strong support might not necessarily be real. Our conventional analysis of aligned gene sequence finds strong and unambiguous support for an asteroid–ophiuroid pairing. However, data partitioning suggests this is most likely an LBA artefact since the data set scoring the most unreliable sites in our alignment strongly support an asteroid–ophiuroid pairing and provide a better fit than either the slow-evolving sites or the complete data to trees displaying this clade. In contrast, the analyses of the homogeneous data set tend to support an ophiuroid + echinoid grouping. This indicates that the signal for this clade is concentrated in the slowly evolving (more reliable) sites (Fig. 2), and is thus likely to represent phylogenetic signal. While this signal is not very strong, this is to be expected given that the signal for this grouping is swamped by other signals in the complete data set. Our signal dissection approach therefore provides a simple means of distinguishing those groupings more likely to be driven by LBA (e.g., asteroid + ophiuroid) from those more likely to represent genuine phylogenetic signal (e.g., echinoid + ophiuroid).

These results help explain why previous molecular analyses have come to different conclusions concerning the interrelationships among eleutherozoan echinoderms. Littlewood et al. (1997)

excluded all sites that could not be unambiguously aligned (thus avoiding the most rapidly-evolving sites), and discovered a weak signal for an echinoid–ophiuroid pairing. In contrast, Janies (2001) and Janies et al. (2011) analysed RNA data using POY (Wheeler et al., 1996; Varón et al., 2010), a technique that carries out tree building and sequence alignment simultaneously on the complete sequence and thus includes regions of highly ambiguous alignment. Initially Janies (2001) found strong support for an asteroid–ophiuroid pairing, and no signal for the echinoid–ophiuroid pairing. Later Janies et al. (2011) showed that class relationships could not be resolved because the outcome was very sensitive to tree search parameters being used. By including poorly aligned (i.e., faster evolving) regions, the direct optimisation approach implemented in POY is much more likely to find support for artifactual clades. Similarly we suspect that Pereske et al.'s (2010) analysis of mitochondrial genome architecture and amino acid sequences, which consistently recovered only one clade comprising asteroids, echinoids and holothurians, is most likely an artefact. Ophiuroids proved to be both very long-branched and highly divergent in genome architecture while crinoids had markedly different nucleotide compositions of protein coding genes.

Confirmation that echinozoans and ophiuroids form a clade will require analysis of many more slowly evolving genes. However, our preliminary results point to this being the only grouping subtended by an historical signal in our data. If correct this has important implications for the morphological evolution of echinoderms. First it confirms that the morphologically similar pluteus larval stages of echinoids and ophiuroids are indeed homologous rather than convergent, as first suggested by Hyman (1955). It also supports the view that neurulation of the radial nerve in echinoids, holothurians and ophiuroids evolved just once, as argued by Heinzeller and Welsch (2001). Finally, the stellate body plan of asteroids and ophiuroids must be plesiomorphic with the globular echinozoan body plan derived from it. The outstanding problem now for palaeontology is to identify whether any of the early fossil asterozoans are potential stem group echinoids + holothurians.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.08.028.

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