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Hemichordates and deuterostome evolution: robust molecular phylogenetic support for a hemichordate + echinoderm clade

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SUMMARY Hemichordates were traditionally allied to the chordates, but recent molecular analyses have suggested that hemichordates are a sister group to the echinoderms, a relationship that has important consequences for the interpretation of the evolution of deuterostome body plans. However, the molecular phylogenetic analyses to date have not provided robust support for the hemichordate + echinoderm clade. We use a maximum likelihood framework, including the parametric bootstrap, to reanalyze DNA data from complete mitochondrial genomes and nuclear 18S rRNA. This approach provides the first statistically significant support for the hemichordate + echinoderm clade from molecular data. This grouping implies that the ancestral deuterostome had features that included an adult with a pharynx and a dorsal nerve cord and an indirectly developing dipleurula-like larva.

INTRODUCTION

Molecular data have provided a new view of metazoan phylogeny, challenging phylogenetic relationships inferred from morphological and developmental data (reviewed in Raff 1998; Zrzavy et al. 1998; Valentine et al. 1999). Molecular studies, particularly those based on the 18S rRNA nuclear gene, have provided support for four major superphyletic clades within the Metazoa: the diploblastic prebilaterians (which includes sponges, ctenophores, cnidarians, and placozoans), the ecdysozoan protostomes (includes arthropods and allies, and nematodes and allies), the lophotrochozoan protostomes (molluscs, annelids, and other spiralians, lophophorates, rhabdocoel flatworms, and some aschelminths), and the deuterostomes (chordates, hemichordates and echinoderms) (e.g., Turbeville et al. 1994; Wada and Satoh 1994; Halanych et al. 1995; Aquinaldo et al. 1997; Cohen et al. 1998; Littlewood et al. 1998; Ruiz-Trillo et al. 1999). However, the relationships of the phyla within these four superphyla are less certain.

The deuterostomes are an informative case in point. While the monophyly of the chordates, hemichordates, and echinoderms is supported by developmental, morphological, and molecular data (Garstang 1928; Berrill 1955; Jefferies 1986; Holland et al. 1991; Turbeville et al. 1994; Wada and Satoh 1994; Halanych et al. 1995; Gee 1997; Peterson et al. 1997; Castresana et al. 1998; Zrzavy et al. 1998), the relationships among these phyla remain debatable. The widely varying body plans displayed by members of these phyla makes assessment of phylogeny from morphological characters difficult and has led to the generation of multiple phylogenetic hypotheses (see Fig. 1). Molecular data, by offering a phylogenetic analysis independent of the major developmental and morphological differences between phyla, could clarify deuterostome relationships.

Previous molecular phylogenies based on the 18S rRNA gene (Holland et al. 1991; Wada and Satoh 1994) and mitochondrial DNA (Castresana et al. 1998) have suggested that hemichordates fall on the echinoderm lineage, forming the Ambulacralia (= Hemichordata + Echinodermata) clade. Combined molecular and morphological data sets (Turbeville et al. 1994; Zrzavy et al. 1998) also support the hemichordate + echinoderm clade. This relationship is gaining acceptance and is informing interpretations of deuterostome evolution and development (e.g., Tagawa et al. 1998; Ogasawara et al. 1999; Peterson et al. 1999a, 1999b). However, while many studies have supported a hemichordate + echinoderm clade, none has provided robust support for the grouping (low bootstrap values suggest that data do not unequivocally support the clade). Deep, phylum-level phylogenies can produce questionable results (Abouheif et al. 1998; Takezaki and Gojobori 1999), so it is important to assess the level of support for the hemichordate + echinoderm clade and to ask whether this grouping could have arisen by an artifact of analysis.

We have evaluated the strength of the molecular phylogenetic signal for a hemichordates + echinoderm clade by reanalyzing the available DNA sequence using the statistical framework of maximum likelihood (including the parametric bootstrap) to ask whether the hemichordate + echinoderm grouping is a significantly better fit to the data than alternative topologies. Our analysis has several advantages over previous molecular phylogenetic studies of the position

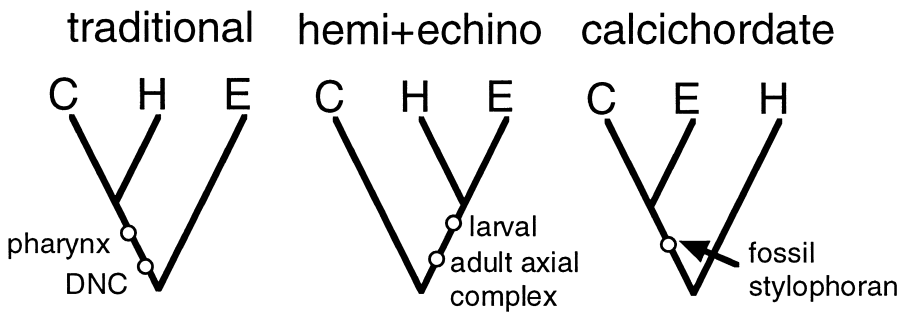

Fig. 1. The "traditional" hypothesis places hemichordates (H) on the chordate (C) lineage, supported by shared U-shaped pharyngeal slits, and possibly a dorsal nerve cord (DNC) and stomachord/notochord-like structure (Basler and Ruppert 1990). However, recent analysis of the expression of the *Brachyury* gene in an enteropneust hemichordate does not lend support to the contention that the stomachord and notochord are homologous (Tagawa et al. 1998; Peterson et al. 1999a). The second hypothesis groups hemichordates and echinoderms (E) based on similar larval forms (Ruppert and Basler 1986; Peterson et al. 1997) and adult axial complex (Basler and Ruppert 1990). A third hypothesis groups echinoderms and chordates to the exclusion of the hemichordates, based on stylophoran fossils which Jefferies (1986) interprets to be chordates possessing the echinoderm-like features of a calcite skeleton and dexiothetism (asymmetrical reduction of structures on the right side).

of hemichordates. We use two independent sequences, conservative alignment of which provides a total of over 5000 base pairs (bp) of both mitochondrial and nuclear DNA sequence data. We use maximum likelihood because, in addition to being more generally accurate and robust than other phylogenetic inference methods (Huelsenbeck 1995; Huelsenbeck and Rannala 1997), maximum likelihood allows the use of reasonably sophisticated models of DNA sequence evolution and provides a tractable statistical framework for assessing the relative support for alternative phylogenetic hypotheses. Parametric bootstrap analysis allows us to assess the probability that the molecular phylogenetic signal that groups hemichordates with echinoderms could have arisen by chance (Huelsenbeck et al. 1996). This hypothesis-testing approach allows us to provide the first strong molecular phylogenetic support for this clade and to confidently reject the "traditional" hypothesis (hemichordates + chordates) and the "calcichordate" theory (echinoderms + chordates; Jefferies 1986) as explanations of these molecular data.

MATERIALS AND METHODS

Evolutionary history can be inferred from DNA sequences by using a substitution matrix to calculate for every site in the sequence the probability that any given phylogenetic tree produced the DNA sequences observed for extant taxa (Felsenstein 1981). On the assumption that all base changes are independent, the overall likelihood of a given phylogenetic tree is the product of probabilities across all sites. The tree that has the highest likelihood is deemed the best fit to the data, and therefore the most probable scenario for the evolution of those sequences given that model of sequence evolution. The likelihood ratio test provides a "universal framework" for comparing the fit of phylogenetic hypotheses to a given dataset; the ratio of the likelihoods of the data maximized under both the null hypothesis and the alternative hypothesis is compared to an expected distribution in likelihood differences given the null hypothesis (Goldman 1993a, 1993b; Huelsenbeck et al. 1996; Huelsenbeck and Rannala 1997). For certain cases, this distribution of expected variance can be assumed (Goldman 1993a, 1993b), but otherwise a null distribution can be generated using Monte Carlo simulation of data (Huelsenbeck et al. 1996; Huelsenbeck and Rannala 1997). This produces an expected distribution of difference in likelihood values between the alternative and null hypotheses if the null hypothesis is true (see Fig. 2). If the difference in likelihoods between the maximum likelihood solution and the null hypothesis is greater than 95% of the expected values, then the null hypothesis can be confidently rejected. This procedure is known as parametric bootstrapping (for a useful review see Huelsenbeck and Rannala 1997).

Data

18S rRNA has been much favored for "deep phylogeny," and consequently it is available for a large range of taxa, although the use of RNA genes in phylogenetic studies is complicated by the heterogenous substitution patterns that are a consequence of the three-dimensional structure of the gene product (Dixon and Hillis 1993; Vawter and Brown 1993; Rzhetsky 1995; Tillier and Collins 1998). The recently sequenced whole mitochondrial genome of an enteropneust hemichordate, *Balanoglossus carnosus*, has now permitted assessment of deuterostome phylogeny using mitochondrial DNA (Castresana et al. 1998). We used a concatenated alignment of 11 protein-coding genes from the mitochondrial genome. Both the 18S rRNA and mitochondrial protein-coding sequences were aligned against sequences from a previous analysis of the origins of metazoans (Bromham et al. 1998; http://evolve.zoo.ox.ac.uk/Alignments/Cambrian.html). Because these alignments included only sites that are informative over much deeper divergences, they are conservative for this analysis.

Analyzing large numbers of simulated data sets can be computationally demanding (in terms of processor time), so the parametric bootstrap is most easily applied to small trees. To focus on the position of the hemichordates with respect to the echinoderm and

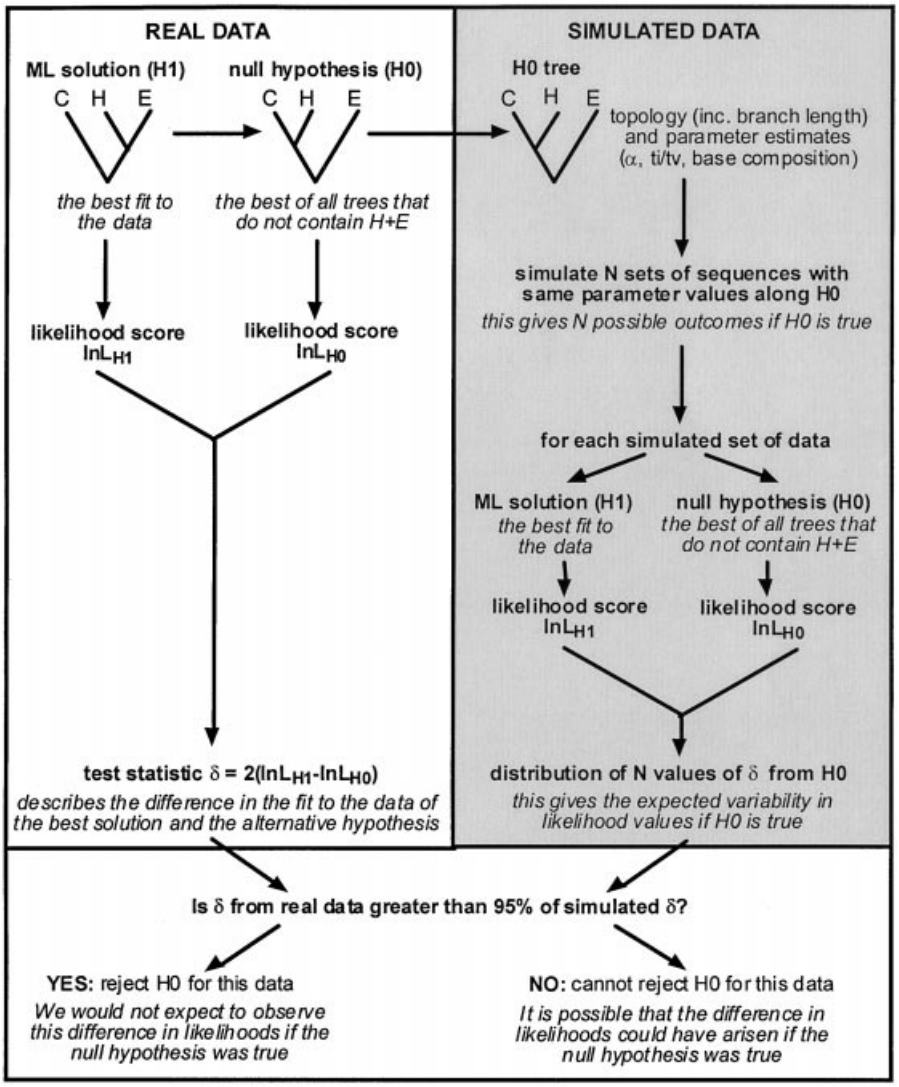

Fig. 2. We use the parametric bootstrap to ask, If the traditional hypothesis was true would we have got the hemichordate + echinoderm grouping by chance? Assessing whether the maximum likelihood (i.e., "best-fit tree"; H1) is a significantly better fit to the data than an alternative topology requires an expected distribution of the difference in likelihood values between the alternative topologies if the null hypothesis is true. We generate a null distribution by Monte Carlo simulation of data under the null hypothesis (using SeqGen; Rambaut and Grassly 1997). If the difference in likelihoods between the maximum likelihood solution and the null hypothesis is greater than 95% of the expected values, then the null hypothesis can be confidently rejected (Huelsenbeck et al. 1996).

chordate lineages, we chose six mitochondrial genomes and nine 18S rRNA sequences to represent these three phyla, plus outgroups (Fig. 3). Given that the hemichordate + echinoderm grouping has been supported by previous analyses using many more taxa (e.g., Holland et al. 1991; Turbeville et al. 1994; Wada and Satoh 1994), including the analysis on which the present study is based which used 40 18S rRNA sequences from 11 phyla including 17 deuterostome sequences (Bromham et al. 1998), we are confident that the result obtained in this study is not simply an artifact of reduced sample size. The two alignments (1538 bp of 18S rRNA and 3688 bp of mitochondrial protein coding genes, first and second codon positions only) were analyzed separately, but a combined alignment of both 18S rRNA and mitochondrial genes for six species was also analyzed (one of the echinoderm sequences being a concatenation of two Asteroidea species, the 18S rRNA of *Asterias amurensis* and the mitochondrial DNA of *Asterina pectinifer*).

Phylogenetic analysis

For each of the alignments, the maximum likelihood tree was found, with an HKY85+Γ substitution model, which allows for uneven base composition and for different rates of transitions and transversions (Hasegawa et al. 1985), with gamma-distributed rates across the sequence (Yang 1994; Yang et al. 1994). We estimated base composition, transition/transversion ratio (ti/tv), and gamma

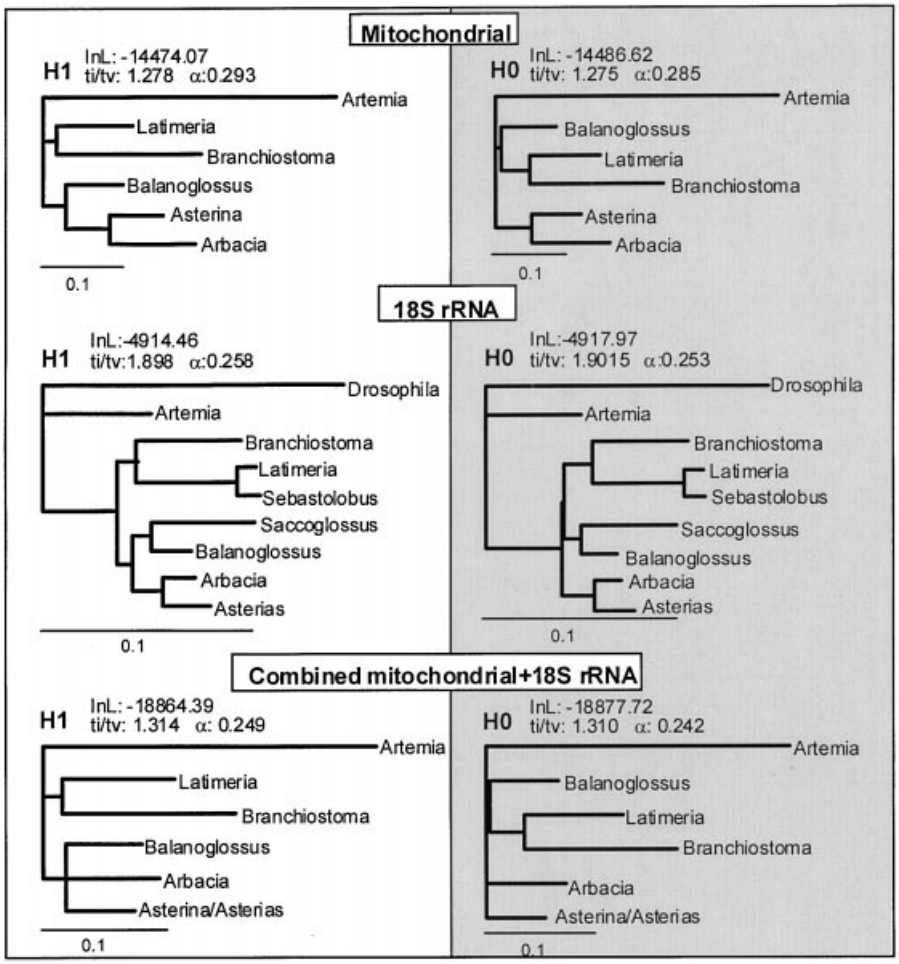

Fig. 3. For both the mitochondrial and 18S rRNA sequences, the maximum likelihood tree (H1) groups the hemichordates (*Balanoglossus carnosus*, *Saccoglossus kowalevskii*) with the echinoderms (*Asterina pectinifer*, *Arbacia lixula*, *Asterias amurensis*), to the exclusion of the chordate clade containing the cephalochordates (*Branchiostoma lanceolatum*) and the vertebrates (*Latimeria chalumnae*, *Sebastolobus altivelis*). Outgroup sequences are an insect (*Drosophila melanogaster*) and a crustacean (*Artemia franciscana*). Each maximum likelihood tree (H1) is compared with the "next best" tree (H0) which does not contain the hemichordate + echinoderm grouping. The log likelihood values (lnL) and estimated transition/transversion ratio (ti/tv) and gamma shape parameters (α) are given for each tree. The scale is in substitutions per site.

shape parameter (α) from the sequence data. Note that because all third codon positions were excluded from the mitochondrial alignment, a codon-based model was not appropriate for this data. The small number of taxa in the mitochondrial alignment made an exhaustive search of all trees possible, but for the larger 18S rRNA tree a heuristic search was performed using TBR branch swapping from an initial neighbor joining tree (see Swofford 1999). The maximum likelihood solution for both 18S rRNA and mitochondrial sequences, and for the combined alignment, groups the hemichordates with the echinoderms (Fig. 3).

To assess whether the maximum likelihood tree is significantly better than alternative topologies, we needed to assess whether the maximum likelihood topology could have arisen by chance if an alternative phylogeny was true. We defined the maximum likelihood tree (which contained the hemichordate + echinoderm grouping) as H1 and defined the null hypothesis, H0, as any tree not containing the hemichordate + echinoderm grouping (such that H0 = not (H1)). H0 was produced by constraining the maximum likelihood solution to exclude trees containing the hemichordate + echinoderm grouping. For both sequences (and the combined alignment), H0 conformed to the traditional hypothesis, placing the hemichordates on the chordate lineage (Fig. 3). We did not explicitly test Jefferies' (1986) calicichordate hypothesis, but given that the tree representing the calcichordate hypothesis has an even lower likelihood, rejection of H0 strongly suggests that the calcichordate hypothesis would also be rejected for this data.

The difference in likelihood between the maximum likelihood solution (H1) and the null hypothesis (H0) is described by the test statistic $\delta = 2(\ln L_{H1} - \ln L_{H0})$. To test whether this observed difference in likelihood between H1 and H0 is significant—that it could not simply be the result of phylogenetic "noise"—a null distribution of expected variation in δ was generated by simulating the evolu-

tion of 100 sets of sequences along the H0 tree, with the same sequence length and estimated gamma shape parameter, transition/transversion ratio, and base compositions as the original data (Fig. 2). Each simulated dataset was analyzed as for the real data, finding the highest likelihood score and the score of the tree constrained to H0 (hemichordates not with the echinoderms), giving a value of δ for each simulated data set. This distribution of 100 δ values represents the expected degree of variation in likelihood scores between H1 and H0 if H0 is true.

RESULTS AND DISCUSSION

For both mitochondrial and 18S rRNA (and the combined alignment) the difference in likelihoods between H1 and H0 is greater than that for any of the simulated datasets, so we can conclude that the likelihood value of H1 would not have arisen if H0 was true. We therefore reject the null hypothesis and conclude that the hemichordate + echinoderm grouping is a significantly better fit to the data than any alternative topology for these data.

Significant support for the hemichordate + echinoderm clade has important implications for understanding the last common ancestor from which echinoderms, hemichordates, and chordates evolved. Consideration of the possible outgroups to the deuterostome clade gives little indication of the features of the ancestral deuterostome, beyond the most basic characters: possession of a eucoelomate bilaterian body plan and probably indirect development (Peterson et al. 1997). Fixing the hemichordate lineage with respect to the root of the deuterostome clade allows inference of the presence of major body plan characterisitics in the ancestral deuterosome on the basis of shared derived characters. Unless the shared characters of hemichordates and chordates (such as the pharynx) have all evolved independently twice, we must conclude that their inclusion in both major arms of the deuterostome tree (chordates on the one hand and hemichordates + echinoderms on the other) is due to their presence in the common ancestor of all deuterostome phyla. Specifically, a hemichordate + echinoderm clade implies that the protodeuterostome exhibited indirect development, where a dipleurula-like larva (a feature present in extant hemichordates and echinoderms; Peterson et al. 1997), metamorphosed into an adult with U-shaped pharyngeal slits and a dorsal nerve cord (features present in extant hemichordates and chordates). This phylogeny is supported by recent interpretations of conserved expression patterns of a number of developmental genes. The hemichordate + echinoderm clade is supported by shared expression patterns of the *Brachyury* gene in the mesocoel (Peterson et al. 1999a, 1999b). The homology of pharyngeal gill slits in both hemichordates and chordates is supported by expression patterns of *Pax1/9* genes in the pharynx of hemichordates, urochordates, and cephalochordates (Holland and Holland, 1995; Ogasawara et al. 1999). Therefore, the hemichordate + echinoderm grouping suggests that the pharynx must have been present in the deuterostome ancestor rather than independently derived in both the chordate and hemichordate lineages.

This suggests that early steps in chordate evolution would have included the acquisition of a notochord-supported muscularized tail, cephalization, and the loss of the dipleurula larval phase and adoption of direct development. The biphasic life history of ascidians and salps appears to be secondarily derived (Wada and Satoh 1994; Wada 1998). Because the deuterostome ancestor must have possessed a pharynx, the chordate postanal muscularized tail complex must have developed independently of the pharynx (Hinman and Degnan, 1999). The hemichordate + echinoderm grouping also implies that the echinoderm adult body plan characters of pentameral symmetry, a water vascular system, an endoskeleton, and ring-shaped nervous system surrounding the mouth that radiates along the arms were not present in the ancestral deuterostome and so these characters must be part of a dramatic autapomorphic transformation in the echinoderm lineage after it diverged from the hemichordate lineage. Fixing the phylogenetic position of the urochordates within the deuterostomes may shed further light on the features of the ancestral deuterostome.

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