Molecular paleobiological insights into the origin of the Brachiopoda

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SUMMARY Most studies of brachiopod evolution have been based on their extensive fossil record, but molecular techniques, due to their independence from the rock record, can offer new insights into the evolution of a clade. Previous molecular phylogenetic hypotheses of brachiopod interrelationships place phoronids within the brachiopods as the sister group to the inarticulates, whereas morphological considerations suggest that Brachiopoda is a monophyletic group. Here, these hypotheses were tested with a molecular phylogenetic analysis of seven nuclear housekeeping genes combined with three ribosomal genes. The combined analysis finds brachiopods to be monophyletic, but with relatively weak support, and the craniid as the sister taxon of all other brachiopods. Phylogenetic-signal dissection suggests that the weak support is caused by the instability of the craniid, which is attracted to the phoronids. Analysis of slowly evolving sites results in a robustly supported monophyletic Brachiopoda and Inarticulata (Linguliformea+Craniiformea), which is regarded as the most likely topology for brachiopod interrelationships. The monophyly of Brachiopoda was further tested with microRNA-based phylogenetics, which are small, noncoding RNA genes whose presence and absence can be used to infer phylogenetic relationships. Two novel microRNAs were characterized supporting the monophyly of brachiopods. Congruence of the traditional molecular phylogenetic analysis, microRNAs, and morphological cladograms suggest that Brachiopoda is monophyletic with Phoronida as its likely sister group. Molecular clock analysis suggests that extant phoronids have a Paleozoic divergence despite their conservative morphology, and that the early brachiopod fossil record is robust, and is not affected by taphonomic factors relating to the late-Precambrian/early-Cambrian phosphogenic event.

INTRODUCTION

Brachiopods are a group of bivalved, sessile, marine invertebrates that dominated Paleozoic seafloors for several hundred million years. They are also one of the first groups to appear in the Cambrian radiation, with the first appearance of phosphatic-shelled and calcareous-shelled brachiopods occurring essentially simultaneously (Ushatinskaya 2008; Zhang et al. 2008; Maloof et al. 2010). This makes them of interest to wider studies of the Cambrian biomineralization event, as it implies either independent evolution of bivalved shells using different mineralogies, or a transition from one biomineral to another, something that is unknown in other taxa such as molluscs, echinoderms, or arthropods, which share temporal origins of biomineralization with brachiopods. Brachiopods are also intriguing because although they are clearly lophotrochozoan protostomes (Field et al. 1988; Halanych et al. 1995; see references in Hausdorf et al. 2010 for recent studies affirming the lophotrochozoan clade) their early development shares many similarities with deuterostomes. This indicates either retention of a plesiomorphic developmental mode in brachiopods (Valentine 1997; Peterson and Eernisse 2001), or extensive homoplasy in developmental characters that were widely believed to robustly delineate deep metazoan phylogeny (Luter and Bartolomaeus 1997). Finally, because of their abundance, especially in the Paleozoic, brachiopods are critical to studies of paleogeography, paleoecology, macroevolutionary trends, and mass extinctions (Gould and Calloway 1980; Veizer et al. 1998; Rode and Lieberman 2004; Butts 2005; Kowalewski et al. 2005; Knoll et al. 2007).

Because the extinct diversity of brachiopods greatly outweighs their extant diversity, most studies of brachiopod evolution have been paleontologically oriented (Williams et al. 1996), and although several competing topologies for brachiopod interrelationships have emerged, all cladistic analyses agree that the phylum is monophyletic (Fig. 1A) (Hennig 1966; Rowell 1982; Popov et al. 1993; Carlson 1995; Holmer et al. 1995; Williams et al. 1996; Zrzavy et al., 1998; Peterson and Eernisse 2001). Many molecular phylogenetic studies, on the other hand, which usually use the small (18S) and large...
evolution. The “soft-shelled” brachiopod strongly influenced paleontological scenarios of brachiopod for example Rota-Stabelli and Telford 2008). Composition heterogeneities among the taxa in question (see transition of the root is possible, especially if there are rate or use only two molluscan outgroups, a simple shift in the position of the root was tested using a molecular paleobiological approach (Peterson et al. 2007) with two different kinds of data. The first data set consisted of the concatenation of seven nuclear housekeeping genes plus the three ribosomal subunits (5.8S, 18S, 28S rDNA) from 72 taxa including five brachiopods representing all three major lineages. A series of phylogenetic analyses were performed using both the complete data set, or analyzing partitions of this data set scoring only relatively fast-evolving, or relatively slowly-evolving, sites to identify sets of phylogenetic relationships likely to represent tree reconstruction artifacts (see Sperling et al. 2009a).

The monophyly of brachiopods was further tested by investigating the presence and absence of specific-microRNA (miRNA) genes. MicroRNAs are short, ~22 nucleotide noncoding genes that regulate protein coding genes by binding with imperfect complementarity to target sites in the 3′ untranslated region of messenger RNAs, affecting their translation (reviewed by Bartel 2009). These genes are an emerging dataset in understanding metazoan phylogenetics as they have several attributes that make them strong phylogenetic characters (Sperling and Peterson 2009). First, the mature 22 nucleotide sequences are likely the most conserved element in the metazoan genome, allowing for relatively simple detection in genomes, small RNA libraries and in northern analysis (Sempere et al. 2006; Wheeler et al. 2009). MicroRNAs work at any scale of metazoan phylogenetics, as lineage-specific microRNAs are continually added through

(28S) ribosomal subunits, have suggested that brachiopods are paraphyletic with the inarticulate brachiopods (the inarticulate and phosphatic-shelled lingulids and discinids—the Linguliformea—and the inarticulate but calcitic-shelled Craniformea) more closely related to the phoronids than they are to the articulate brachiopods (Rhynchonelliformea) (Cohen and Weydmann 2005; Santagata and Cohen 2009) (Fig. 1B). Importantly, the morphological and ribosomal trees propose identical topologies, but different rootings (Fig. 1C)—morphological studies root the tree on the phoronid branch, whereas the molecular studies root the tree on the rhynchonelliform branch. Given that the ribosomal studies of Cohen and colleagues (Cohen et al. 1998; Cohen 2000; Cohen and Weydmann 2005; Santagata and Cohen 2009) generally use only two molluscous outgroups, a simple shift in the position of the root is possible, especially if there are rate or composition heterogeneities among the taxa in question (see for example Rota-Stabelli and Telford 2008).

This ribosomal topology, and particularly the implication that phoronids are derived, shell-less brachiopods, has strongly influenced paleontological scenarios of brachiopod evolution. The “soft-shelled” brachiopod Lingulosaccus nuda from the Lower Cambrian Mural Formation of Canada, for instance, was described as a potential stem-group phoronid in the process of losing its shell (Balthasar and Butterfield 2009). This shell loss may have been part of a broader trend in early brachiopod evolution to lose or modify an ancestral phosphatic biomineralization (Skovsted et al. 2009b; Balthasar et al. 2009) in a transition toward either calcitic biomineralization (Balthasar 2007) or toward a “soft-shelled” form (Holmer and Caron 2006; Balthasar and Butterfield 2009). As an alternative hypothesis, the presence of nonbiomineralizing phoronids within the brachiopods could provide evidence for theories of brachiopod origins that considered the brachiopod shell as convergent, arising as many as seven times independently from infaunal lophophorate ancestors during a transition to an epifaunal lifestyle (Valentine 1973; Wright 1979; Gorjansky and Popov 1986). The implications of brachiopod paraphyly with respect to phoronids has also influenced ideas regarding the comparative embryology of both groups (Freeman 2000, 2003; Santagata and Zimmer 2002).

Here, the monophyly of brachiopods and the position of the root was tested using a molecular paleobiological approach (Peterson et al. 2007) with two different kinds of data. The first data set consisted of the concatenation of seven nuclear housekeeping genes plus the three ribosomal subunits (5.8S, 18S, 28S rDNA) from 72 taxa including five brachiopods representing all three major lineages. A series of phylogenetic analyses were performed using both the complete data set, or analyzing partitions of this data set scoring only relatively fast-evolving, or relatively slowly-evolving, sites to identify sets of phylogenetic relationships likely to represent tree reconstruction artifacts (see Sperling et al. 2009a).

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evolutionary history, and once these genes are added, they are only rarely secondarily lost in most taxa (Sempere et al. 2006; Sperling et al. 2009b; Wheeler et al. 2009). Finally, the probability of evolving the same ~ 22 nucleotide sequence, within the genomic context of an ~ 70 nucleotide sequence that can fold back upon itself into a canonical microRNA hairpin structure, is vanishingly small, meaning that microRNAs are unlikely to evolve convergently (Sperling and Peterson 2009).

To test the results of the traditional molecular phylogenetic analysis, small RNA libraries were built from the three main brachiopod lineages, sequenced with next-generation sequencing technology, and combined with previously published data from other lophotrochozoan lineages (including phoronids) to investigate the monophyly of brachiopods with respect to all other phyla. The microRNA-based phylogeny agrees with the molecular phylogenetic analysis in suggesting that brachiopods are monophyletic. These molecular data, combined with new insights from the rich brachiopod fossil record, provide a more comprehensive understanding of the early evolution of the clade.

**MATERIALS AND METHODS**

**Data collection**

Specimens of *Terebratalia transversa*, *Phoronis vancouverensis*, *Phoronopsis harmeri*, *Carinoma mutabilis*, and *Tubuladus polymorphus* were collected at the Friday Harbor Marine Laboratory, San Juan Island, WA, USA. *Lingula anatina*, collected from Amani Island, Japan, was kindly provided by K. Endo (U. of Tokyo). *Glottidia pyramidata* and *Phoronis architecta* were purchased from Gulf Specimens Marine Supply (Panacea, FL, USA). The seven nuclear housekeeping genes used in the molecular clock study of Peterson et al. (2004) namely aldolase, methionine adenosyltransferase, ATP synthase β chain, catalase, elongation factor 1 α, triosephosphate isomerase, and phosphofructokinase (were sequenced from these eight taxa following the protocol described in Sperling et al. (2007). These sequences have been deposited in GenBank under accession numbers (HQ833664–HQ833700). Sequences for other lophotrochozoan taxa were taken from previously published reports. The *Phoronis muelleri* sequences here in initial phylogenetic analyses, and two genes, triosephosphate isomerase and elongation factor 1 α, appeared as potential annelid contaminants in single gene trees; consequently this taxon was not included in our analysis.

These data were then concatenated with data for ribosomal 5.8S, 18S, and 28S ribosomal genes. Data for 10 taxa were taken directly from the Mallatt et al. (2010) alignment, an extensive dataset of full-length or nearly full-length ribosomal 5.8S, 18S, and 28S genes from 197 metazoan taxa, with the alignment constructed manually with reference to conserved structural diagrams.

Ribosomal data for the remaining taxa were downloaded from the NCBI Genbank website and manually aligned to the Mallatt et al. (2010) sequences. For the brachiopods and phoronids, ribosomal data was only used if sequenced from the same species as sequenced for the nuclear housekeeping genes; for other lophotrochozoan taxa chimaeras at the generic level were permitted when data for the same species was not available. The resulting matrix included 35 lophotrochozoan taxa, with seven nuclear housekeeping genes (2049 amino acids total) and three ribosomal genes (4682 nucleotides total). These lophotrochozoan sequences were then combined with data for 15 edysosoan and 22 deuterostome outgroups, which were added to increase the number of calibration points for the molecular clock analyses and the number of possible monophyletic groups in the Slow-Fast analysis (see below and supporting information). After the removal of minor indels, the amino acid matrix was 88% complete and the ribosomal matrix was 76% complete. As the relationships between outgroup organisms are essentially static across analyses, lophotrochozoan outgroups are not figured; complete trees can be found in supporting information Figs. 1–10.

**Phylogenetic analyses**

Extensive model testing of a dataset with metazoan-wide sampling of the seven nuclear housekeeping genes used here indicated that the CAT-GTR model implemented in Phylobayes (Lartillot et al. 2009) was the overall best-fitting model (Sperling et al. 2009a). This was followed, in terms of overall goodness of fit, by CAT, mechanistic GTR, and finally by the empirical GTR matrices such as WAG (all models including G correction). This order is likely to be a general trend for amino acid datasets (Lartillot et al. 2009). The GTR+G model was found to be the overall best-fitting model for the nucleotide data, as assessed using the AIC in MrModeltest (Nylander 2004). The two data partitions (rDNA and proteins) were analyzed independently and as a concatenated data set. The rDNA data was analyzed using GTR+G in MrBayes, whereas the protein data set was analyzed using Phylobayes under the CAT-GTR+G model. The concatenated (rDNA plus proteins) alignment was analyzed under mixed models using MrBayes v. 3 (Ronquist and Huelsenbeck 2003). For all MrBayes analyses we performed two runs of four independent chains, each using two independent mechanistic GTR+G models (one for the rDNA partition and one for the protein partition). All Bayesian analyses were run until convergence was reached (with the burnin period being analysis-dependent). Convergence was tested for the Phylobayes analyses using bppcomp (which is part of the Phylobayes software) while for the MrBayes analyses likelihood values were plotted against each other to evaluate whether a likelihood plateau was reached, and the average standard deviation of split frequencies of trees obtained in the two runs was monitored. Trees were rooted on the protostome-deuterostome split.

**Tree reconstruction artifacts**

The combined rDNA and protein data set was analyzed using Minimum Evolution (with observed distances and no G correction). This method is highly sensitive to long-branch attraction artifacts and might be expected to be misled when the data are saturated and lineage-specific substitution rates are heterogenous.
Support for the nodes in the ME tree was estimated using the bootstrap, and comparison between our Bayesian trees and the ME tree were used to pinpoint sets of taxa that might be grouped together (in the ME tree) because of systematic artifacts.

Both the rDNA and the protein data sets were partitioned into sets of homogeneously evolving and heterogeneously evolving sites according to their evolutionary rate, following the procedure of Sperling et al. (2009a). Full details of this modified Slow-Fast (Brinkmann and Philippe 1999) analysis are described in the supporting information. For each data type (proteins and rDNAs), characters were partitioned in two data sets. The first (herein “rate heterogeneous”) contained all sites in the fourth quartile based on evolutionary rate, as well as all invariant sites. The fast-evolving characters (fourth quartile) are prone to homoplasy when addressing questions at this phylogenetic depth, and when added to the invariant sites, between-site rate heterogeneity is increased to high levels. Because of the extreme rate variation and presence of homoplasy-prone sites, this dataset is expected to be difficult to analyze and likely to support phylogenetic artifacts even when analyzed using well-fitting, parameter-rich models (see Sperling et al. 2009a). The second data set (herein “rate homogeneous”) contained all the sites in the first through third quartiles (but excluding the invariant sites), which are phylogenetically more-reliable, rate-homogeneous characters, and more likely to support relationships that represent historical signal (see Sperling et al. 2009a; Rota-Stabelli et al. 2011).

The rate homogeneous and rate heterogeneous rDNA data set were analyzed using the GTR+G model, whereas the homogeneous and heterogeneous amino acid data sets were analyzed using the CAT-GTR model. After that, the two homogeneous data sets, and the two heterogeneous data sets, were combined to generate two final mixed data sets. The first included all the homogeneous characters (both rDNA and proteins), whereas the second contained all the heterogeneous characters. Both data sets were analyzed in MrBayes under mixed models using a separate GTR+G model for both the nucleotide partition and the protein partition.

**Molecular clock analysis**

Relaxed molecular clock analyses were performed following the protocol of Sperling et al. (2010). All analyses were performed relaxing the clock assumption using the CIR model, an autocorrelated model, which we previously showed (Sperling et al. 2010) fit this data set better than uncorrelated models like those implemented in the software BEAST (Drummond and Rambaut 2007). In order to use all available metazoan calibration points, an additional 29 sponge, cnidian, and nonmetazoan outgroups were added to the dataset. Clock analyses used a fixed topology based on the results of the combined protein+ribosomal analysis of the entire dataset analyzed under the conditions outlined above (results for additional basal metazoans were congruent with those in Sperling et al. 2009a). Because the phylogenetic position of Novocrania could not be determined with certainty, this taxon was removed from the molecular clock analyses. Branch lengths for this fixed topology (shown in supporting information Fig. 11) were reestimated under the CAT-GTR model using only the protein alignment. We used a total of 24 calibration points spread phylogenetically throughout Metazoa and temporally from the Miocene to Cryogenian. All calibration points and the source of their justification are listed in supporting information Table S1. Twelve chains were run, and their results averaged. Analyses were 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. All analyses were run using soft bounds, and experiments were performed to test the effect of different levels of bound-relaxation on the recovered ages. To perform such experiments we calculated divergence times allowing 5% (default in Phylobayes), 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. All analyses were performed using a prior root age of 1000 Ma and a standard deviation of 100 Ma (see Peterson et al. 2008 for discussion of this root prior). 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 this prior on our divergence times.

**microRNA phylogenetics**

Small RNA libraries were constructed from the brachiopods *T. transversa*, *G. pyramidata*, and *Novocrania anomala*. The *Novocrania* specimens were collected at Kristineberg Marine Station, Sweden and kindly provided by M. Obst (Göteborgs Universitet); collection of all other specimens as above. Library construction followed the protocol outlined in Wheeler et al. (2009). Briefly, ~ 200 μg of total RNA was run on a polyacrylamide gel, and the RNA size fraction between 18 and 25 nucleotides was excised and eluted. Linkers were ligated to the 5′ and 3′ ends and used for reverse transcription and polymerase chain reaction (PCR) amplification. Libraries were barcoded, pooled, and a total of 424,832 reads from these libraries were generated using 454 sequencing technology (Margulies et al. 2005) at the Yale Center for Genomics and Proteomics.

These reads were then analyzed using the program miRMiner (Wheeler et al. 2009), a program designed to identify conserved and novel microRNAs from small RNA libraries. Small RNA reads from each taxon were initially searched using miRMiner against miRBase release 14. After the identification of known microRNAs and removal of rRNA, tRNA, and mRNA fragments, candidate novel microRNAs were selected for validation. All candidates capable of being detected by northern analysis that potentially supported either the monophyly of brachiopods or the topology of Cohen and colleagues (Cohen and Weydmann 2005; Santagata and Cohen 2009) were investigated. The discovery of additional microRNAs, and microRNAs supporting brachiopod internal relationships, are outside the scope of our validation process and will require genomic data.

Potential candidates were validated using genome-walking and northern analysis. Northern analysis determines whether the mature gene product is expressed at the correct ~ 22-nucleotide size, whereas genome-walking demonstrates that the genomic context of the mature sequence folds into a canonical microRNA hairpin structure (Ambros et al. 2003). Genome-walker libraries were constructed using the GenomeWalker Universal Kit (Clontech,
Mountain View, CA, USA) from G. pyramidata and Terebratulina septentrionalis (purchased from Gulf of Maine Marine Supply, Pembroke, ME, as high-quality genomic DNA could not be isolated from collected samples of T. transversa). PCR conditions, cloning, and sequencing of genome-walker products followed Wheeler et al. (2009), with the exception that genome-walking proceeded in both directions as it was unknown whether the mature sequence was located on the 5' or 3' arm of the microRNA. Northern analyses were also conducted as described in Wheeler et al. (2009), with 10 μg of total RNA per organism. In addition to the organisms sequenced for small RNA libraries, RNA obtained from specimens of Laqueus californianus (kindly provided by J. Barry; MBARI), Mytilus californianus (kindly provided by C. Tanner; SIO), Phoronopsis, Nereis, and Cerebratulus were also used in comparative northern analysis. Northruns on tissue dissections of nephridia, mantle, muscle, gonad, and lophophore from T. transversa were performed to investigate possible tissue specificity of novel microRNAs.

RESULTS

Molecular phylogenetics

Combined analysis

The combined analysis of nuclear housekeeping genes plus ribosomal genes finds brachiopods to be monophyletic, with the phoronids as their sister group (Fig. 2A; supporting information Fig. S1). The sister-group relationship between brachiopods and phoronids has a posterior probability of 1.0, as does the monophyly of phoronids, but the monophyly of brachiopods is only moderately supported, with a posterior probability of 0.82. Within the Brachiopoda, the sampled lingulids and articulate species are recovered as reciprocally monophyletic, each with a posterior probability of 1.0. The craniid Novocrania is then found as the sister group to the lingulids+articulates, but with relatively weak support for that node, a posterior probability of 0.67. Thus although this analysis agrees with the morphological analyses in supporting brachiopod monophyly (Fig. 1A) it supports a different topology with craniids as the most basal branch of brachiopods.

Identification of potential phylogenetic artifacts

To ask if there are confounding systematic artefacts associated with this change in topology, we first analyzed the combined protein and rDNA data sets with Minimum Evolution. In contrast to the Bayesian analyses, this analysis found phoronids nested within the Brachiopoda as sister to the inarticulates (Fig. 2B; supporting information Fig. S2), which is the same topology of Cohen and Weydmann (2005) and Santagata and Cohen (2009).

Comparison of trees from the Slow-Fast analysis can help arbitrate between the results of the Bayesian and Minimum Evolution analyses and identify clades that are likely to be robust, with their signal derived from slowly-evolving and rate-homogenous sites, from those that are likely to be affected by systematic biases, with signal derived from fast-evolving sites that are more prone to homoplasy, or from poor model fit due to the artificially exacerbated rate heterogeneity (all individual analyses shown in supporting information Figs. S3–S10). In the “homogeneous sites only” analysis of the combined dataset (Fig. 2C; supporting information. Fig. S3), Brachiopoda is still found to be monophyletic, but with much stronger statistical support (PP = 1.0) than in the full analysis. Further, the craniid, rather than being placed as the sister group to all other brachiopods, now groups with the inarticulates with moderate support (PP = 0.85), consistent with the rooted topology shown in Fig. 1A.

In contrast to the homogeneous sites, in the “heterogeneous-sites only” analysis of the combined dataset the phoronids are found within the brachiopods and strongly supported (PP = 0.98) as the sister group to Novocrania (supporting information Fig. S6). In summary, analyses of both the “homogeneous sites only” and “heterogeneous sites only” datasets result in strongly supported, but differing, topologies, with the slow-evolving and rate-homogeneous sites supporting brachiopod monophyly, and the fast-evolving and rate-heterogeneous sites supporting brachiopod paraphyly with the phoronids as sister group to Novocrania. The inclusion of the heterogeneous sites changes not only the topology of the tree, but the position of the root as well. This suggests that the most likely topology and root of the brachiopod tree is the result obtained from the homogenous sites analysis (Fig. 2C), and the tree diagrammed schematically in Fig. 1A—with both Brachiopoda and Inarticulata as monophyletic clades.

microRNA phylogenetics

Because the essential difference between the two alternative rooting positions concerns the monophyly of the brachiopods, we sought to test this hypothesis by investigating a different data set—that of miRNAs. Given how they evolve through time (see “Introduction”), miRNAs have the potential to be powerful arbitrators between competing hypotheses. This is especially true for establishing the most likely position of the root, as microRNA gene analysis is not subject to rate or compositional heterogeneity biases, but instead is based on the acquisition of a novel character(s). To test between these two phylogenetic alternatives, small RNA libraries were constructed and sequenced (see supporting information Table S2) for three brachiopods and potential novel miRNAs identified with miRMiner (Wheeler et al. 2009). Screening potential microRNA candidates by northern analysis and genome-walking revealed two novel microRNA families that are shared among brachiopods. These two genes are found in the small RNA library reads of all three brachiopods investigated.
(Fig. 3A), but not in the phoronid \textit{P. architecta} or in any other taxon's genome or small RNA library investigated to date (\textit{miRBase} v. 16). Northern analysis shows that they are indeed expressed at the correct size (Fig. 3, Band D) as expected for a \textit{bona fide} miRNA (Ambros et al. 2003), and that transcripts can be detected in the RNA of additional brachiopods (\textit{Terebratulina} and \textit{Laqueus}) but not in the RNA of a different phoronid, \textit{P. harmeri}.

To further confirm that these expressed sequences are in fact miRNAs, the flanking sequence for one of these microRNAs was genome-walked from \textit{G. pyramidata} and \textit{T. septentrionalis}. The mature microRNA is on the 5' arm in each case, and the sequences fold into a hairpin structure with the requisite free energy values (Fig. 3E). This microRNA therefore has evidence of both expression and biogenesis (Ambros et al. 2003) and has been accessioned into \textit{miRBase} as \textit{miR-5045}. The second gene is likely a microRNA given its presence in small RNA libraries targeting products of Dicer cleavage, evolutionary conservation, and the expression as a 22mer, but without genomic data cannot be accessioned into \textit{miRBase} and is referred to as brachiopod candidate 1 (\textit{BC1}).

Unlike nuclear housekeeping genes and ribosomal DNA, microRNAs are not independent of phenotype, and as some have restricted expression domains (e.g., Christodoulou et al. 2010), it is possible that the loss of certain structures may result in loss of the microRNAs controlling tissue specificity and homeostasis in those structures. For example, Sperling et al. (2009b) interpreted the absence of annelid-specific microRNAs in sipunculans as a primitive absence. However, Dordel et al. (2010) suggested that these microRNAs may function in segmentation or chaetogenesis in annelids, and that the absence of these genes in sipunculans was a secondary loss coupled to loss of these structures. Similarly, if phoronids were nested within brachiopods (Cohen and Weydmann 2005; Santagata and Cohen 2009), and the brachiopod-specific microRNAs are only expressed in the mantle and are involved in biomineralization processes, the loss of the shell in phoronids could also result in loss of these microRNAs. Northern run on RNA from tissue dissections of \textit{Terebratulina} demonstrate that the novel microRNAs \textit{miR-5045} and \textit{BC1} are expressed ubiquitously, including in the lophophore (Fig. 3C), and thus their absence in the phoronids cannot be explained by loss of a morphological character such as the mantle.
Molecular clock

Because our topology is robust, in the sense that both our homogenous site analysis (Fig. 2C) and the miRNAs (Fig. 3) suggest that brachiopods are monophyletic, we then sought to ask if the fossil record of brachiopods is robust by using a molecular clock. Molecular divergence estimates for lophophorate nodes are listed in Table 1 and shown on Fig. 4. The results for these nodes from all chains are shown in supporting information Table S4, and the results for all other nodes are shown in supporting information Fig. S11 and supporting information Table S5. The divergence of the sampled phoronids is in the Carboniferous at 336 Ma (with a 95% posterior distribution of 270–403 Ma), and the divergence between P. architecta and Phoronopsis at 322 Ma (257–383). The divergence between the brachiopods and phoronids is estimated to be mid-Ediacaran, at 578 Ma (546–612). The estimated divergence between the Linguliformea and Rhynchonelliformea just predates the Precambrian–Cambrian boundary at 547 Ma (501–587). If the inarticulates are monophyletic (e.g., Fig. 2C) or the sister group of the articulate brachiopods investigated but not in the library of the phoronid Phoronis architecta or any other metazoan taxon’s genome or small RNA library. BC1 is likely a microRNA given its presence in small RNA libraries designed to sequence products of Dicer cleavage, evolutionary conservation, and expression of the mature form at ~22 nucleotides, but without genomic data cannot be accessioned in miRBase. BC1 was detected by Northern analysis at the correct ~22 nucleotide size in the articulate brachiopods Terebratalia and Terebratulida and the inarticulate brachiopods Glottidia and Novocrania, but not in the two phoronids, the bivalve mollusc Mytilus, annelid Ne-reis or nemertean Cerebratulus, consistent with 454 sequencing results of small RNA libraries. (C) Northern analysis of miR-5045 and BC1 on tissue dissections from the brachiopod Terebratalia, demonstrating that these microRNAs are expressed ubiquitously and are not tissue-specific. (D) Northern analysis of miR-5045 in the articulate brachiopod Laqueus, demonstrating that the mature form is detected at the correct ~22 nucleotide size. Only enough Laqueus RNA was recovered for one blot. All positive bands in (B) and (C) are expressed at the same ~22 nucleotide size but only the band is shown. (E) miR-5045 was cloned from the Glottidia and Terebratulina genome-walker libraries and is found on the top arm in each case. All structures have the requisite free-energy values and show the mature sequence (in bold) within two nucleotides of the loop.

Table 1. Mean divergence estimates, with 95% posterior distribution in parentheses, for the nodes depicted in Fig. 4

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Age</th>
</tr>
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<tbody>
<tr>
<td>Brachiopoda (Lingulida + Terebratulida)</td>
<td>547 (501–587)</td>
</tr>
<tr>
<td>Lingulida (Lingula + Glottidia)</td>
<td>191 (128–281)</td>
</tr>
<tr>
<td>Terebratulida (Terebratulida + Terebratulina)</td>
<td>449 (355–518)</td>
</tr>
<tr>
<td>Phoronida (P. architecta + P. vancouverensis)</td>
<td>336 (270–403)</td>
</tr>
<tr>
<td>Brachiopoda + Phoronida</td>
<td>578 (546–612)</td>
</tr>
</tbody>
</table>

As taxon sampling is not complete across clades, these ages only correspond to the nodes as defined in this table (e.g., crown-group Lingulida may not be defined by the last common ancestor of L. anatina and G. pyramidata).
and Rhynchonelliformea is 547 Ma, closely predating the first appearance of stem-group members of each of these clades in the Ediacaran (indicated by arrow), indicating a close concordance between molecular and fossil ages for this divergence. The divergence between brachiopods and phoronids is at 578 Ma, providing an estimate of \( C_{24} \) banian (indicated by arrow), indicating a close concordance between molecular and fossil ages for this divergence. The divergence between brachiopods and phoronids is at 578 Ma, providing an estimate of \( C_{24} \) banian (indicated by arrow), indicating a close concordance between molecular and fossil ages for this divergence. The divergence between brachiopods and phoronids is at 578 Ma, providing an estimate of \( C_{24} \) banian (indicated by arrow), indicating a close concordance between molecular and fossil ages for this divergence. The divergence between brachiopods and phoronids is at 578 Ma, providing an estimate of \( C_{24} \) banian (indicated by arrow), indicating a close concordance between molecular and fossil ages for this divergence. The divergence between brachiopods and phoronids is at 578 Ma, providing an estimate of \( C_{24} \) banian (indicated by arrow), indicating a close concordance between molecular and fossil ages for this divergence.

Ediacaran in brown, Cambrian in green, post-Cambrian Paleozoic in purple, Mesozoic in pink, Cenozoic in yellow.

Fig. 4. Molecular divergence estimates for brachiopods and phoronids estimated under the autocorrelated CIR model and using soft bounds (5% default in PhyloBayes) for the calibration points. Mean ages and 95% posterior distribution listed in Table 1; these values represent averages of 12 independent chains (see supporting information Table 4). Horizontal gray bars represent 95% posterior distribution. Extant phoronids appear to have a Paleozoic divergence despite their conservative morphology. The mean age for the divergence between the Linguliformea interval of each considered calibration point caused negligible changes to estimated lophophorate ages, and in all but one case still recovered divergence times that lay within the 95% confidence interval of the analysis run under the default 5% relaxation level (supporting information Table S3). Finally, changing the root prior age did not significantly affect our recovered divergence times (supporting information Table S3).

**DISCUSSION**

**Brachiopod monophyly**

All morphological cladistic analyses to date have supported brachiopod monophyly (Hennig 1966; Rowell 1982; Carlson 1995; Holmer et al. 1995; Williams et al. 1996; Zrzavy et al., 1998; Nielsen 2001; Peterson and Eernisse 2001). Both molecular datasets investigated here, the traditional (protein plus rDNA) and the microRNAs, support a monophyletic Brachiopoda. Although the statistical support for brachiopod monophyly in the combined analysis (Fig. 2A) is not especially high, the comparison of our Slow-Fast trees clearly illustrate that this is driven by the instability of *Novocrania*, which is artifactually attracted to the phoronids. Brachiopods are found to be monophyletic in all three “homogeneous sites only” analyses, with strong support in the analysis of the combined dataset (Fig. 2C). Two microRNAs, miR-5045 and BC1, also support brachiopod monophyly. These two microRNAs are highly expressed, and are not found in the deeply sequenced library of *P. architecta* nor in the RNA of *P. harmeri* in northern analysis (Fig. 3, A and B), nor any other investigated metazoan taxon. They show ubiquitous expression in tissue dissections (Fig. 3C) and are therefore not tissue-specific genes (e.g., expressed only in the mantle) that might have been lost if the shell was lost along the phoronid stem-lineage.

We previously argued that congruence of independent lines of evidence on the same tree topology is the strongest possible proxy of phylogenetic accuracy (Pisani et al. 2007; Sperling et al. 2009a; Rota-Stabelli et al. 2011). Morphological cladograms, recent ribosomal and EST phylogenetic studies (Hausdorf et al. 2010; Mallatt et al. 2010), the combined nuclear housekeeping gene plus ribosomal DNA study here, and the microRNA data, all suggest that brachiopods are monophyletic with phoronids as their likely sister taxon. This level of congruence, combined with the demonstration that brachiopod paraphyly is likely the result of uncorrected systematic biases, strongly argues in favor of brachiopod monophyly.

As discussed above, our preferred topology (Fig. 1A) and the topology of Cohen and Weydmann (2005) and Santagata and Cohen (2009) (Fig. 1B) underlie the same unrooted tree: the difference is the placement of the root (Fig. 1C). Both those ribosomal gene studies found that the articulate brachiopods have significantly faster rates of molecular evolution than the phoronids or inarticulates. Thus, the unrooted topologies of Cohen and colleagues are likely to be correct, but the accelerated rate of evolution for the articulate brachiopods caused them to be attracted toward the two molluscan outgroup sequences, and the root placed between the articulates and the phoronids+inarticulates. An incorrect rooting in the ribosomal trees is consistent with our observation that the topology of Cohen and colleagues can be reproduced by analyzing our complete data set using Minimum Evolution (Fig. 2B), but not using better performing methods, that is, under conditions that are sensitive to long-branch attraction. Rooting problems are likely to become more frequently observed in metazoan phylogeny as the field moves from incongruence caused by random error to that caused by systematic error (Jeffroy et al. 2006; Rota-Stabelli and Telford 2008; Rota-Stabelli et al. 2011).
### Brachiopod interrelationships

Morphological cladistic analyses of brachiopod interrelationships are in agreement on the monophyly of the three classes (Rhynchonelliformes, Linguliformes, Craniiformes) but disagree on their relationships. Some analyses find the inarticulates monophyletic (Fig. 1A; Rowell 1982; Carlson 1995; Williams et al. 1996), whereas others support the Calcitsa hypotheses with the calcitic-shelled taxa as sister groups with respect to the phosphatic Linguliformes (Hennig 1966; Holmer et al. 1995). These results, though, are sensitive to outgroup choice (Carlson 1995; Williams et al. 1996) and in the dataset of Carlson (1995) all three possible topologies for clade-level relationships are only separated by one step (supporting information Fig. S12). Carlson (2001) notes that the craniids share approximately the same number of characters with linguliforms and rhynchonelliforms, with the problem being one of establishing polarity with respect to divergent outgroups. Embryological and neuroanatomy study on craniids also reveal a mixture of traits that make their placement uncertain (Nielsen 1991; Freeman 2000; Altenburger and Wanninger 2010). In our molecular phylogenetic analysis the craniids behave as a "rogue taxon" with little statistical resolution at the base of Brachiopoda (Fig. 2A), and thus our analysis mirrors extant morphology and the fossil record in suggesting that the radiation of the three main brachiopod lineages is rapid and represents a difficult phylogenetic problem. Because Novocrania appears to be affected by systematic biases (as evidenced by its attraction to the outgroup taxa in the "heterogeneous sites only" analyses—supporting information Fig. S6 and S7), the position of craniids as the sister group to the lingulids as found in the "homogenous sites only" analysis of the combined dataset is provisionally supported as their most likely placement (Fig. 2C), as this is the analysis least likely to be affected by phylogenetic artifacts. This result, the monophyly of Inarticulata, occurs within the context of a relatively well-resolved brachiopod tree, suggesting that there is strong signal for this topology once the fast-evolving sites have been removed.

### Molecular clock

With a more robust brachiopod phylogeny, it is then possible to apply molecular clock methodologies to address questions such as the temporal length of the brachiopod stem-lineage during which brachiopod-specific characters evolved, as well as provide a test of the early brachiopod fossil record. Although brachiopods are generally assumed to have a complete fossil record, each clade needs to be investigated individually, as Precambrian taphonomic pathways may differ substantially from those of the Phanerozoic (e.g., Sperling et al. 2010). As this study was focused on testing the relative completeness of the early brachiopod fossil record using an independent dataset, no internal brachiopod calibration points were used.

The divergence between lingulids and articulate brachiopods is here dated to 547 Ma (95% posterior distribution = 501–587 Ma; Fig. 4; Table 1). There are likely stem-group representatives to the rhynchonelliforms such as Salangyolina (Holmer et al. 2009) and the kutorginids (Zhang et al. 2007) in the Lower Cambrian, by at least the Atdabanian, if not the Tommotian (Ushatinskaya 2008). Abundant and well-documented stem-group linguliforms have also been documented from the Atdabanian Chengjiang fauna in South China (Hou et al. 2007 and references therein). This therefore represents a close concordance between the molecular divergences between the Rhynchonelliformes and the Linguliformes and the first appearance of stem-members of each clade. Given current age estimates for the Atdabanian and Tommotian (Maloof et al. 2010), the molecular estimates predate the fossil first appearances by ~20 Ma. While a 20 million year difference is not insignificant, considering the possible errors on fossil age estimation, that the molecular divergence estimate represents a mean age within a confidence interval (with the first appearance of crown-brachiopods clearly falling within this interval), and that genetic divergences are expected to predate the first appearance of fossils recording that divergence (see Fig. 1 of Benton and Donoghue 2007), it is clear these ages are congruent. Thus, this study finds, using an independent test, that the brachiopod fossil record is a robust chronicle of the clade's early evolution, and demonstrates that the first appearance of phosphatic brachiopods was not significantly influenced by the widespread phosphogenesis at the Precambrian–Cambrian boundary (Cook 1992) that affected aspects of fossil preservation during this time period (Porter 2004; Brasier and Callow 2007). More importantly, the close concordance between the molecular divergence estimates and the fossil ages, without the use of brachiopod calibration points, gives confidence that the molecular ages for other lophophorate nodes will be relatively accurate.

Our analysis places the divergence between Terebratulina and Terebratulina in the early Paleozoic, at 449 Ma. This is older than current hypotheses (Lee et al. 2006; Lee and MacKinnon 2006; MacKinnon and Lee 2006), which suggest that the divergence of these living forms occurred in the Permian-Triassic. It is, though, consistent with older theories that placed the divergence of the Terebratulida and the Terebratulida, and the evolutionary origin of "long loop" forms, in the Early Devonian (Muir-Wood et al. 1965; Stehli 1965). Many of the Paleozoic long-loop forms (the "loop" being a biomineralized support for the lophophore on the dorsal valve) such as in Cryptonelloidea are considered to be convergent on the long-loop Terebratulida (which are thought to have arisen around the Permian-Triassic boundary) (MacKinnon and Lee 2006; Lee and MacKinnon 2006). This is primarily due to ontogenetic differences in loop formation, although the authors do note that the adult loop...
structures in Paleozoic and post-Paleozoic forms are in fact quite similar. The early Paleozoic molecular divergence found in this study suggests that the appearance of long-looped forms at this time might mark the emergence of the Terebratellidina lineage rather than convergent evolution in loop morphology.

The divergence between the dated phoronids at 336 Ma indicates the clade has an ancient history, a result also found by Santagata and Cohen (2009). While phoronids may have cryptic morphological diversity (especially at the larval stage; Santagata and Zimmer, 2002), their traditional classification into only 12 species in two genera belies the conservative nature of the group. These deep molecular divergences are therefore surprising, and imply that modern phoronids are essentially living fossils, experiencing little morphological change over long periods of geologic time, but with soft bodies and therefore no actual fossil record.

The divergence between brachiopods and phoronids is dated to 578 Ma, which when compared with the age for the brachiopod crown group provides an estimate of ~30 million years for the brachiopod stem-lineage and the origin of brachiopod synapomorphies such as the mantle and shell. It has been suggested that a group called the tommotiids, which are a component of the Lower Cambrian “small shelly fossil” assemblage, may form the paraphyletic stem group to the Brachiopoda (Holmer et al. 2008; Skovsted et al. 2008, 2009a, b; Balthasar et al. 2009; Kouchinsky et al. 2010) and can thus help delineate the sequence in which crown-group synapomorphies arose (e.g., Smith 2005). The discovery of more complete scleritomes is expected to yield important insights into the origin of the brachiopod shell and the sequence of character addition in the critical ~30 million year interval of brachiopod evolution along the stem-lineage that data from extant organisms are unable to polarize.

CONCLUSIONS

The interrelationships of brachiopods, and more broadly, the relationships of the lophotrochozoan phyla, represent difficult phylogenetic problems as each are rapid, ancient radiations. Nonetheless, larger datasets and phylogenetic-signal dissection reveal robustly supported relationships and help identify those clades that are likely to result from systematic biases. The two molecular datasets investigated here, a traditional molecular phylogenetic analysis of seven nuclear housekeeping genes combined with three ribosomal genes, and the presence/absence of specific microRNAs, find brachiopods to be monophyletic. Within brachiopods, the inarticulates (Linguliformea+Craniformea) are likely monophyletic, although this is obscured by the artifactual attraction of the cranid toward the phoronid outgroups. If this result, the monophyly of inarticulates, is validated, it would require at least two transitions from phosphatic to calcitic biomineralization if phosphatic biomineralization is ancestral for brachiopods, as suggested by some studies of the tommotiids (Balthasar et al. 2009; Skovsted et al. 2009b). This is intriguing as such wholesale shifts in biomineral type cannot be convincingly demonstrated in any other metazoan lineage (Bengtson and Runnegar 1992).

Although a congruent picture of brachiopod systematics appears to be emerging, all datasets, including morphology, microRNAs and traditional molecular systematics have either low resolving power, or non-trivial homoplasy that must be recognized and corrected. This study is the first to include data from nuclear housekeeping genes from the three main groups of brachiopods, and as taxon sampling plays an important role in phylogenetics (Graybeal 1998; Hillis et al. 2003), it is expected that wider sampling will be useful in more robustly assessing brachiopod phylogeny. The sequencing of a brachiopod genome, which can then be used to discover microRNAs delineating internal brachiopod relationships, is also likely to be informative. The new molecular data can then be integrated with the detailed morphological and paleontological studies that have characterized, and continue to characterize, the study of this phylum, and used to address questions related to biomineralization, developmental patterns, and the morphological evolution of a clade that dominated seafloors for over half of Phanerozoic history.

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Fig. S4. Bayesian analysis of the rate homogenous ribosomal sites under the GTR+G model in Phylobayes. Supports are posterior probabilities. Due to very long branch lengths, some of the branch lengths within the insects are depicted with breaks in scale.

Fig. S5. Bayesian analysis of the rate homogenous protein sites under the CAT-GTR+G model in Phylobayes. Supports are posterior probabilities.

Fig. S6. Bayesian analysis of combined rate heterogeneous sites (ribosomal + protein), under mixed models in MrBayes. The protein and ribosomal partitions were each modeled using a separate GTR+G model. Supports are posterior probabilities.

Fig. S7. Bayesian analysis of the rate heterogeneous protein sites under the CAT-GTR+G model in Phylobayes. Supports are posterior probabilities. Note that unlike all other analyses, it is not possible to root between protostomes and deuterostomes as these clades are not recovered as reciprocally monophyletic.

Fig. S8. Bayesian analysis of the rate heterogeneous ribosomal sites under the GTR+G model in Phylobayes. Supports are posterior probabilities. Lophotrochozoan phyla are not labeled due to the paraphyly or polyphyly of many phyla. Due to very long branch lengths, some of the branch lengths within the insects are depicted with breaks in scale.

Fig. S9. Bayesian analysis of the complete protein dataset (2049 amino acids) under the CAT-GTR+G model in Phylobayes. Supports are posterior probabilities. Lophotrochozoan phyla are not labeled due to the paraphyly or polyphyly of many phyla. Due to very long branch lengths, some of the branch lengths within the insects are depicted with breaks in scale.

Fig. S10. Bayesian analysis of the complete ribosomal dataset under the GTR+G model in MrBayes. Supports are posterior probabilities. Lophotrochozoan phyla are not labeled due to the paraphyly or polyphyly of many phyla. Due to very long branch lengths, some of the branch lengths within the insects are depicted with breaks in scale.

Fig. S11. Fixed topology used in molecular clock analyses. This topology was derived from an analysis of the full protein plus ribosomal dataset for the 72 taxa in this study, combined with an additional 29 sponge, cnidarian and non-metazoan outgroups in order to use all available metazoan calibration points. This analysis was conducted in MrBayes using mixed models, with a separate mechanistic GTR + G model used for both the protein-coding and ribosomal partitions. Branch lengths for the protein-coding dataset were re-estimated for this topology under the CAT-GTR+G model in Phylobayes and used in subsequent molecular clock analyses. This time tree shows the results of the chain (chain 11) whose mean age for the base of Brachiopoda was closest to the average of all 12 chains.

Fig. S12. Hypotheses of brachiopod and phoronid relationships and the number of morphological steps as calculated from the morphological matrix of Carlson (1995). A) In the Inarticulata/Articulata hypothesis the two inarticulate groups, the calcitic-shelled Craniiformea (represented in this study by Novocrania) and the phosphatic-shelled Linguliformea (here Glotidia and Lingula) are sister groups with respect to the calcitic-shelled articulate brachiopods, the Rhynchonelliformea (here Terebratulina and Terebratulina). B) In the Calcita hypothesis, the calcitic-shelled brachiopods (Craniiformea + Rhynchonelliformea) form a monophyletic group with respect to the phosphatic-shelled Linguliformea. C) This topology lacks a formal name in the literature and is herein described as “Craniiformea basal,” with the Craniiformea as the sister group to Linguliformea + Rhynchonelliformea. D) The topology resulting from some ribosomal DNA studies (Cohen and Weydmann, 2005; Santagata and Cohen, 2009) places the phoronids within the brachiopods as the sister group to the inarticulates, e.g. Craniiformea + Linguliformea. The number of steps for each hypothesis in the morphological matrix of Carlson (1995), as calculated in MacClade using the Sipuncula as outgroup, is shown in italics under the name of each hypothesis. Other outgroups or combinations of outgroups resulted in different total tree lengths but no change in the relative number of steps between hypotheses.

Table S1. Molecular clock calibration points.
Table S2. 454 reads.
Table S3. Results of sensitivity tests for selected nodes testing the relaxation of soft bounds on calibration points and the use of a deeper root prior.
Table S4. Divergence estimates and Min-Max 95% posterior distribution for each of the 12 chains run in the molecular clock analysis for the lophophorate nodes. Results for all other nodes are shown in Supplementary Figure 11.
Table S5. As visualization on one figure of such a large tree, the divergence estimates, and the 95% posterior distribution for all nodes is difficult, the raw data for chain 11 is also included in .tre format in Supplemental Table 5. See Supplemental Figure 11 for fixed topology in graphical format.

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