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Where's the glass? Biomarkers, molecular clocks, and microRNAs suggest a 200-Myr missing Precambrian fossil record of siliceous sponge spicules

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ABSTRACT

The earliest evidence for animal life comes from the fossil record of 24-isopropylcholestane, a sterane found in Cryogenian deposits, and whose precursors are found in modern demosponges, but not choanoflagellates, calcareans, hexactinellids, or eumetazoans. However, many modern demosponges are also characterized by the presence of siliceous spicules, and there are no convincing demosponge spicules in strata older than the Cambrian. This temporal disparity highlights a problem with our understanding of the Precambrian fossil record – either these supposed demosponge-specific biomarkers were derived from the sterols of some other organism and are simply retained in modern demosponges, or spicules do not primitively characterize crown-group demosponges. Resolving this issue requires resolving the phylogenetic placement of another group of sponges, the hexactinellids, which not only make a spicule thought to be homologous to the spicules of demosponges, but also make their first appearance near the Precambrian/Cambrian boundary. Using two independent analytical approaches and data sets – traditional molecular phylogenetic analyses and the presence or absence of specific microRNA genes – we show that demosponges are monophyletic, and that hexactinellids are their sister group (together forming the Silicea). Thus, spicules must have evolved before the last common ancestor of all living siliceans, suggesting the presence of a significant gap in the silicean spicule fossil record. Molecular divergence estimates date the origin of this last common ancestor well within the Cryogenian, consistent with the biomarker record, and strongly suggests that siliceous spicules were present during the Precambrian but were not preserved.

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INTRODUCTION

Central to understanding the Cambrian explosion is distinguishing between evolutionary origins versus geological first appearances (Runnegar, 1982). This is especially true of sponges, generally considered one of the more basal, if not the most basal, of animal lineages (Telford, 2009). Although triplobast remains and traces are well documented in Ediacaran deposits (Fedonkin & Waggoner, 1997; Martin *et al.*, 2000), sponges fossils are notoriously rare in the Ediacaran (Gehling & Rigby, 1996; Brasier *et al.*, 1997) and do not make an unequivocal appearance before the Cambrian (Xiao *et al.*, 2005). When they do appear all major fossilizable lineages are present including the hexactinellids and the demosponges, in addition to extinct groups including the archaeocyaths, and more enigmatic taxa such as *Eiffelia* (Botting & Butterfield, 2005) and the chancellorids (Sperling *et al.*, 2007).

The difficulty in interpreting this fossil record is that both molecular divergence estimates (Peterson *et al.*, 2008), as well as biomarkers (Love *et al.*, 2009), place the origin of demosponges well into the Cryogenian, at least 100 Ma before they make their first appearance in the fossil record (Bengtson *et al.*, 1990; Xiao *et al.*, 2005). This early appearance is somewhat expected given their placement on the metazoan tree of life, but creates problems when trying to account for the tens to hundreds of millions of years of the missing poriferan fossil record, specifically the absence of highly preservable siliceous mega-spicules (Xiao *et al.*, 2005). One solution is to argue that the first appearance of sponges in the Cambrian represents the origin of sponge crown groups (Brocks &

Butterfield, 2009). Thus, for demosponges, because the living members are characterized by the possession of siliceous spicules, and siliceous spicules are demonstrably absent in rocks before the latest Ediacaran, the origin of the crown group must be Cambrian in age (Fig. 1).

Hexactinellid sponges, however, also make their first appearance in the Lower Cambrian (Xiao *et al.*, 2005) and produce a spicule similar, and most likely homologous, to that of demosponges (Leys, 2003; Uriz *et al.*, 2003; Kaluzhnaya *et al.*, 2007; Muller *et al.*, 2008a,b). If demosponges are monophyletic (Philippe *et al.*, 2009; Sperling *et al.*, 2009), then not just crown-group demosponges (c.f. Brocks & Butterfield, 2009), but total-group demosponges (stem + crown) can be no older than the Cambrian if the first appearance of spicules is taken to represent their evolutionary origin (Fig. 1A). This would lead to the conclusion that demosponges cannot be the source of the 'demosponge-specific' biomarkers in the Cryogenian, and these sterols must have been derived from some other organism during this time interval, possibly even unicellular metazoan forerunners (Brocks & Butterfield, 2009). An important implication of this hypothesis is that these sterols must have then been secondarily lost in all metazoan lineages *except* demosponges (Fig. 1A). The retention of these sterols in modern demosponges is then a shared primitive character, and not a shared derived character as parsimoniously assumed by Love *et al.* (2009).

An alternative scenario is that if hexactinellids were actually highly derived demosponges, which has received some, albeit weak, support from a few molecular phylogenetic analyses (Cavalier-Smith & Chao, 2003; Nichols, 2005; Dohrmann *et al.*, 2008), then crown-group Demospongiae need not be restricted to the Cambrian, contra Brocks & Butterfield (2009). This is because crown-group demosponges would be characterized by the *absence* of siliceous spicules, with spicules evolving within demsoponges after the keratosid demosponges split from the siliceous spicule-bearing clades (Fig. 1B). Thus, the origin of the crown group cannot be constrained by the skeletal record, allowing it to have evolved much earlier in time, and possibly in the Cryogenian. The most parsimonious interpretation of these data is then that stem and possibly crown-group demosponges were the source of the sterols, with hexactinellids secondarily losing them sometime after the Cambrian (Fig. 1B).

Clearly the source of the supposed demosponge-specific biomarkers is of major importance to our understanding of the timing of metazoan origins. Because the C_{30} sterols that are the source of 24-isopropylcholestane are absent from all other potential groups – including choanoflagellates (Kodner *et al.*, 2008), calcareous sponges (Love *et al.*, 2009), and hexactinellids (Blumenberg *et al.*, 2002), resolution of this problem requires more robustly ascertaining the interrelationships among the major sponge groups, in particular the

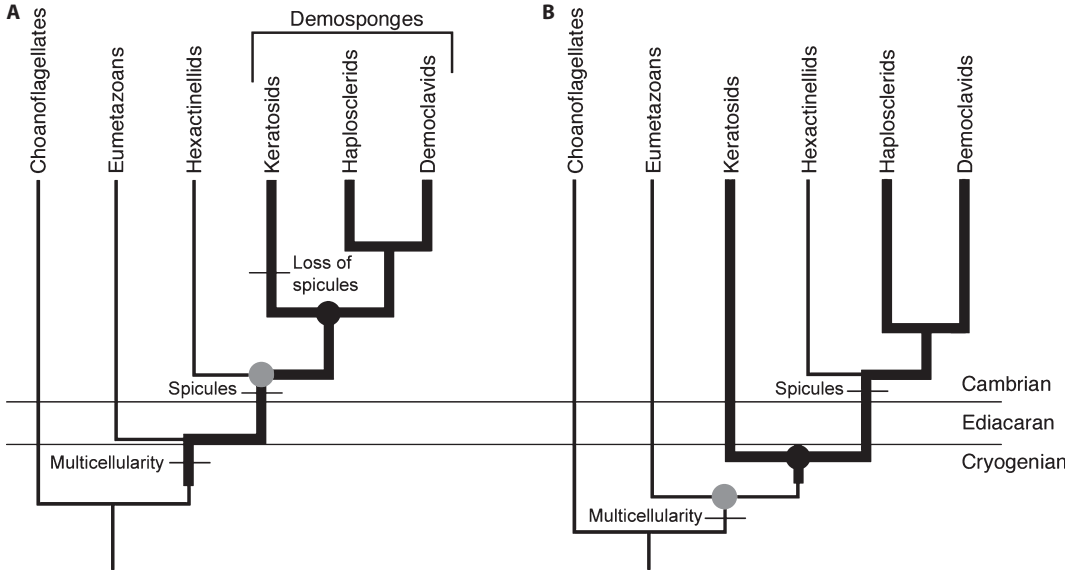

Fig. 1 Two competing views with respect to demosponge-specific biomarkers (bold lines) and the first appearance of spicules, assuming that the first appearance of fossil spicules represents their actual evolutionary appearance. Crown-group Demospongiae is indicated with the black circle and crown-group Silicea with the gray circle. (A) If demosponges are monophyletic, and hexactinellids are their sister taxon, then total-group Demospongiae would necessarily be restricted to the Cambrian, assuming spicules are homologous between hexactinellids and demosponges (with a secondary loss in the keratose sponges). Importantly, the source of the 'demosponge-specific' biomarkers (Love *et al.*, 2009) could not be demosponges, but possibly from metazoan unicellular ancestors (Brocks & Butterfield, 2009). This would necessitate, though, numerous secondary losses of the sterol source of the biomarkers in eumetazoans and other sponge taxa including hexactinellids. (B) If, however, hexactinellids are the sister taxon to the spiculate demosponges, then spicules could have evolved within demosponges, obviating the need to restrict the demosponge crown group to the post-Ediacaran (Brocks & Butterfield, 2009). This would allow for a potentially extensive Precambrian history of demosponges, and thus crown-group demosponges could be the source of the 'demosponge-specific' sterol, but this would necessitate its secondary loss in the hexactinellids.

relationship that exists between demosponges and hexactinellids. Here, using two independent approaches and data sets, phylogenetic analysis of nuclear protein-coding genes and a new molecular data set, the presence and absence of specific microRNA genes, we show that demosponges are monophyletic, and that hexactinellids are the sister taxon of the demosponges (Silicea; Gray, 1867). Using a molecular clock, we estimate that hexactinellids diverged from demosponges over 750 Ma, and that the major radiation of demosponge taxa, including the spicule-bearing ones, all occurred well before the Cambrian, sometime in the early Ediacaran. Thus, the absence of siliceous spicules during the Ediacaran and Cryogenian cannot reflect the first appearance of the respective crown groups (contra Brocks & Butterfield, 2009), but instead reflects a massive failure to preserve siliceous spicules during the Precambrian. All available data strongly suggest that demosponges are indeed the source of the biomarkers, suggesting that metazoan multicellularity has its roots in the Cryogenian, and that the appearance of siliceous spicules at or near the base of the Cambrian does not represent their evolutionary origins, but instead reflects their geological first appearances due to the opening of a new taphonomic window near the Precambrian–Cambrian boundary.

MATERIALS AND METHODS

microRNA libraries and analysis

Small RNA libraries were built as described (Wheeler *et al.*, 2009) from the demosponges *Dysidea camera* (purchased from Gulf Specimens Marine Supply, Panacea, FL), *Clathria* (*Microciona*) *prolifera*, and *Suberites* sp. (both purchased from Marine Biological Laboratories, Woods Hole, MA, USA), the homoscleromorph sponge *Oscarella carmela* (a gift from Dr Scott Nichols, UC Berkeley), the calcarean sponge *Sycon* sp. and the ctenophore *Pleurobrachia* sp. (both collected at Friday Harbor Laboratories, San Juan Islands, WA, USA), and the hexactinellid sponge *Aphrocallistes vastus* (a gift from Dr Sally Leys, University of Alberta). Libraries were barcoded, pooled, and 518 886 reads were generated using 454 sequencing technology (Margulies *et al.*, 2005) at the Yale Center for Genomics and Proteomics Sequencing Facility. The resulting reads were analyzed using the program miRMiner (Wheeler *et al.*, 2009). A previously constructed genome-walker library for *A. vastus* (Rosengarten *et al.*, 2008) was used to clone hairpin structures from that organisms' genome using the mature sequence as the gene-specific primer and the PCR and cycling conditions as described (Wheeler *et al.*, 2009).

Molecular phylogenetics and divergence estimates

We extended the taxonomic sampling of Sperling *et al.* (2009) by adding a new calcarean sponge species, *Leucetta chagosensis* (sequences downloaded from NCBI trace archives), and performed new phylogenetic analyses of this extended data set using Phylobayes (Lartillot & Philippe, 2004). Phylogenetic analyses were performed under the best fitting amino acid substitution model (CAT + G with GTR exchange rates; CAT–GTR) – see Sperling *et al.* (2009) for details. Leaf stability measures (Thorley & Wilkinson, 1999) were then used to test the phylogenetic stability of the species in our data set.

Relaxed molecular clock analyses were then performed under the best-fitting CIR clock model (Lepage *et al.*, 2007) to estimate divergence times of clades found in our phylogenetic analysis (Fig. 2). Both the CIR and the lognormal models relax the molecular clock assumption by implying rate autocorrelation among the branches of the considered phylogeny (Lepage *et al.*, 2007). Because Bayes factors (as implemented in Phylobayes) showed that the difference in fit to the data strongly discriminates in favor of autocorrelated models [and against uncorrelated ones – ln(BF) in (3.7453–10.8722)], here we only used an uncorrelated clock model (the uncorrelated gamma multipliers model – Drummond *et al.*, 2006; see also Lepage *et al.*, 2007), within the context of a sensitivity analysis; i.e. to test whether our results were model dependent.

Because the Hexactinellida were long branched and could potentially influence age estimates, we performed two sets of molecular clock analyses. In the first set, all species in Fig. 4 were included, as well as all eumetazoans included in Sperling *et al.* (2009). In the second set of analyses, the Hexactinellida were excluded. To calibrate our molecular clock analyses, the 14 bilaterian calibration points of Peterson *et al.* (2008) were used. In addition, a maximum age constraint of 713 Myr was imposed for the crown-group demosponges (Love *et al.*, 2009). However, all calibration points were considered soft, i.e. a proportion of the prior probability density for each calibration point was assigned to lie outside the min–max interval. Because molecular clock analyses using soft bounds require a prior on the root node age, a divergence estimate of 1 Ga (±250 Ma SD) was assigned to the Fungi–Holozoa split (see Peterson *et al.*, 2008 for justifications). This then necessitated the exclusion of *Dictyostelium discoideum* from our data set, as the divergence time of this taxon from the remaining species in our data set is unknown. Analyses were performed with no data to assess the joint priors on our calibration points.

Sensitivity analyses were also performed to test: (i) the effect of relaxing the soft bounds; (ii) the effect of using suboptimal relaxed molecular clock methods; and (iii) the effect of eliminating the root prior in a hard bound analysis. To test the effect of relaxing the soft bounds, divergence times were calculated by allowing a total of 5%, 10%, 25%, and 50% of the prior probability density of each calibration point to lie outside the min–max interval. To test the effect of using

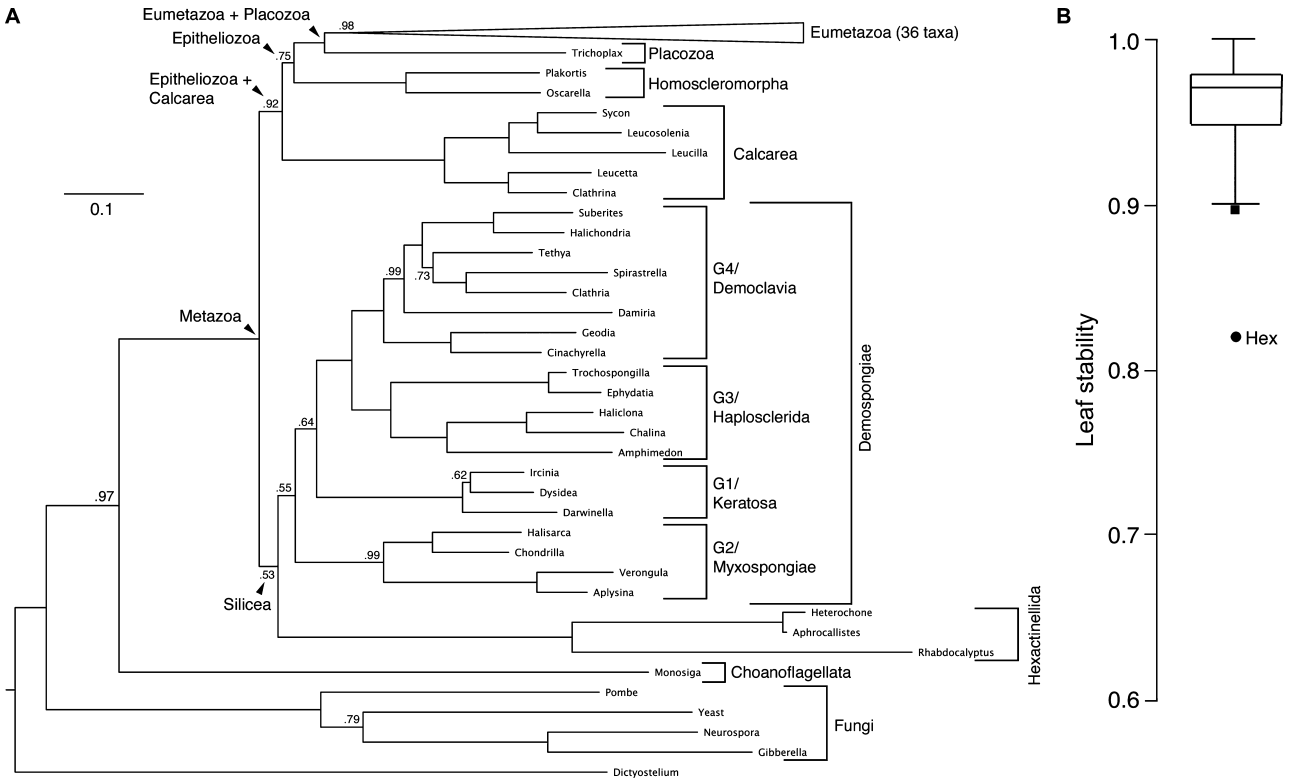

Fig. 2 (A) Bayesian phylogenetic analysis of seven nuclear housekeeping genes (2057 amino acids) under the overall best-fitting CAT + G with GTR exchange rates (CAT–GTR) model (see Sperling *et al.*, 2009 for details of model testing). This analysis uses the same taxon sampling as Sperling *et al.* (2009) but with the addition of the calcinean *Leucetta chagosensis*. Support values are posterior probabilities, and all nodes have a support of 1.0 except where indicated. Sponges are recovered as paraphyletic with the homoscleromorphs and calcareous sponges recovered as successive sister lineages to Placozoa + Eumetazoa. Hexactinellids are found as sister taxon to a monophyletic Demospongiae, but with very low support. A leaf stability analysis of these data (B) find the hexactinellids to be the most unstable of all taxa considered, consistent with other phylogenetic analyses that have recovered a diversity of discordant positions (see text), and highlighting the difficulty of placing problematic taxa such as hexactinellids with traditional molecular phylogenetic analyses.

alternative relaxed molecular clock models, results obtained under the CIR (best fitting) model were compared to results obtained using the second and the third best-fitting models. These models were respectively the lognormal clock model of Thorne and colleagues (Thorne *et al.*, 1998; Thorne and Kishino, 2002), and the uncorrelated gamma multipliers model of Lepage *et al.* (2007). In all soft bound analyses, prior probabilities on calibration points were modeled using the default parameters for the Couchy distribution in Phylobayes. Finally, to test the effect of the prior on the root node, hard bound analyses (which do not require a prior age for the root node) were also performed. These analyses were performed with or without the 713 Ma maxima for the crown Demospongiae.

RESULTS

Molecular phylogenetics of Silicea

The topology found by Sperling *et al.* (2009) under the overall-best-fitting CAT + G with GTR exchange rates (CAT–GTR) model did not change with the inclusion of a second calcinean sponge, and *Leucetta* was recovered as the sister taxon to the other calcinean *Clathrina* with perfect support (Fig. 2A). As in Sperling *et al.* (2009), we find the hexactinellids as the sister group to a monophyletic Demospongiae (Fig. 2A). However, support for this hypothesis is very low with a posterior probability of 0.53. This instability is due, at least in part, to the high rate of molecular evolution (long branch) and mild compositional heterogeneity in the hexactinellid sequences causing them to often be attracted to alternative positions under less-optimal evolutionary models (Sperling *et al.*, 2009). Leaf-stability values, a measure of a taxon's phylogenetic instability (Thorley & Wilkinson, 1999), found the hexactinellids to be the most unstable of all taxa studied (Fig. 2B). This is consistent with the diversity of results obtained by previous phylogenetic analyses, which placed hexactinellids as the sister taxon to all other animals (Kruse *et al.*, 1998), the sister group to demosponges (Collins, 1998; Adams *et al.*, 1999; Medina *et al.*, 2001; Philippe *et al.*, 2009; Sperling *et al.*, 2009), within demosponges (Cavalier-Smith & Chao, 2003; Nichols, 2005;

Dohrmann *et al.*, 2008), and even as the sister group to Bilateria (Haen *et al.*, 2007).

microRNAs

Because of these rate and compositional heterogenities between the sequences of hexactinellids and most other sponge taxa, the accurate and precise placement of the hexactinellids with respect to other metazoan taxa using traditional sequence-based molecular phylogenetics will be perennially difficult. Nonetheless, a robust phylogeny is a crucial prerequisite for molecular clock analyses (Smith & Peterson, 2002). Thus, we tested our phylogeny (Fig. 2) using an independent data set, the presence and absence of specific microRNAs. microRNAs (miRNAs) are small, approximately 22 nucleotide RNA genes that negatively regulate protein-coding genes by binding to target sites in the 3′-untranslated region (Bartel, 2009). They are also an emerging new data set for metazoan phylogenetics (Sperling & Peterson, 2009). miRNAs show four properties that make them potentially very useful tools to resolve metazoan phylogenetic interrelationships. First, miRNAs have very few nucleotide substitutions to the mature sequence, allowing them to be easily recognized in genomes or small RNA libraries (Wheeler *et al.*, 2009). Second, novel miRNAs arise continually through geologic time in all eumetazoan lineages examined to date (Sempere *et al.*, 2006; Sperling & Peterson, 2009; Wheeler *et al.*, 2009). Third, they are only rarely secondarily lost once incorporated into gene regulatory networks (Sempere *et al.*, 2006; Sperling & Peterson, 2009; Wheeler *et al.*, 2009; Sperling *et al.*, 2009). And finally, they are almost impossible to evolve convergently due to the improbability of evolving the same approximately 22 nt sequence within the context of an approximately 70 nt sequence that folds into the canonical microRNA hairpin structure (Sperling & Peterson, 2009).

Initial investigations found that eight miRNAs are shared between the two haplosclerid demosponges *Haliclona* and *Amphimedon*, and these genes were not found in any other taxon investigated to date including choanoflagellates (Grimson *et al.*, 2008; Wheeler *et al.*, 2009). These miRNAs share several features with eumetazoan miRNAs including the extreme conservation of the mature gene sequence (Wheeler *et al.*, 2009). Previous Northern analyses suggested that at least six of these eight miRNAs were restricted to demosponges, with the possible exception of miR-2019, as transcripts of this miRNA were detected in the hexactinellid *Rhabdocalyptus*. No miRNAs were detected in the homoscleromorph *Oscarella* or the calcarean *Leucilla* (Wheeler *et al.*, 2009). Thus, these data suggest that both Silicea and Demospongiae are monophyletic.

To confirm these initial suggestions, we built and explored the small RNA complements of several metazoan taxa including the keratose demosponge *Dysidea*, the democlavid (Sperling *et al.*, 2009) sponges *Suberites* and *Clathria*, the hexactinellid *Aphrocallistes*, the homoscleromorph sponge *Oscarella*, the calcarean *Sycon*, and the ctenophore *Pleurobrachia*. Our data show that all eight microRNAs appear to be primitive for the clade Haplosclerida + Democlavia, as reads for all eight were found in the two democlavids, with the exception of the lowly expressed mir-2017 in *Clathria* (Fig. 3A, Table 1). Two of these eight miRNAs, miR-2018 and miR-2020, are restricted to this clade and were not found in the small RNA library of the keratose sponge *Dysidea*. This result is consistent with the emerging demosponge phylogeny, which places the aspiculate Keratosa and Myxospongiae as a sister group or sister grade to the Haplosclerida + Democlavia (Borchiellini *et al.*, 2004; Lavrov *et al.*, 2008; Sperling *et al.*, 2009). Thus, six of these eight miRNAs appear to be primitive for demosponges, and consistent with this observation, transcripts of three of these miRNA genes (miR-2014, -2016, and -2021) were detected by Northern analysis in the myxosponge *Halisarca* (Wheeler *et al.*, 2009) (Fig. 3B).

Importantly, hundreds of reads of miR-2019, but none of the other demosponge-specific miRNAs, were found in the small RNA library of the hexactinellid *Aphrocallistes*, again consistent with the Northern results (Wheeler *et al.*, 2009) (Fig. 3B). To validate that this small RNA sequence emanates from a miRNA locus (Ambros *et al.*, 2003), we amplified the surrounding region from the genome of *Aphrocallistes* (Fig. 3C). Similar to miR-2019 of *Amphimedon* (Grimson *et al.*, 2008; Wheeler *et al.*, 2009), the mature sequence lies on the 5′ arm, and similar to all known demosponge-specific miRNAs, but unlike eumetazoan miRNAs, the mature gene product is more than two nucleotides away from the loop (Wheeler *et al.*, 2009). This, coupled with the Northern results (Wheeler *et al.*, 2009) that show the presence of miR-2019 transcripts in the hexactinellid *Rhabdocalyptus* (Fig. 3B), confirms the presence of miR-2019 in hexactinellids.

Seven of these eight silicean-specific microRNAs were not found in any other small RNA library sequenced in this study, including the homoscleromorph *Oscarella* and the ctenophore *Pleurobrachia* (Table 1). The *Sycon* library did show a single read for one of the demosponge-specific microRNAs, mir-2016, but at orders-of-magnitude lower expression levels compared to the demosponges (Table 1). Thus, this gene is almost certainly not part of the *Sycon* genome, and is likely to be an epibiont contamination, perhaps by a demosponge larva. This is consistent with the Northern data that showed that miR-2016 was not detected in the calcisponge *Leucilla* (Wheeler *et al.*, 2009; Fig. 3B). Thus, because these silicean-specific microRNAs are also not found in genomes or small RNA libraries from choanoflagellates, cnidarians, placozoans, or bilaterians (Grimson *et al.*, 2008; Wheeler *et al.*, 2009), these data strongly suggest a sister-group relationship between Hexactinellida and Demospongiae, the monophyly of Demospongiae, and the placement of the keratose demosponges outside Democlavia + Haplosclerida, as found with our protein-coding gene tree (Fig. 2A).

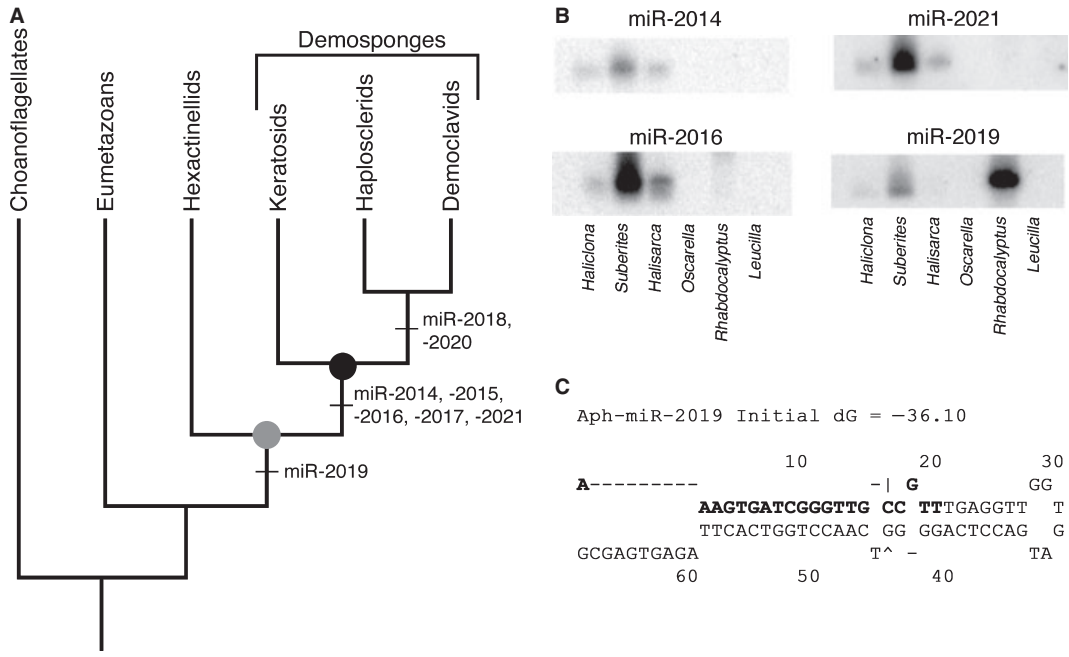

Fig. 3 microRNAs in silicean sponges. (A) Phylogenetic distribution of the eight previously identified miRNAs in haplosclerid demosponges (Grimson *et al.*, 2008; Wheeler *et al.*, 2009) as ascertained by 454 small RNA library sequencing. Note that there are no indications of secondary losses except for the apparent absence of the lowly expressed miR-2017 in the democlavid *Clathria*. (B) Northern analyses of selected miRNAs for the haplosclerid *Haliclona*, the democlavid *Suberites*, and the myxosponge *Halisarca*, as well as other non-demosponge taxa including the homoscleromorph *Oscarella*, the hexactinellid *Rhabdocalyptus*, and the calcarean *Leucilla* (from (Wheeler *et al.*, 2009). Note that only the miRNA miR-2019 is detected outside of the demosponges whereas the other three miRNAs are only detected in the three demosponge taxa, consistent with the 454 data [see (A) and Table 1]. (C) Secondary structure of the miR-2019 gene from the hexactinellid *Aphrocallistes*. The mature sequence cloned from the small RNA library is shown in bold.

Molecular divergence estimates

Although the initial study of Peterson *et al.* (2008) suggested a relatively deep origin for demosponges, their study had only a limited number of sponge taxa included, and did not include any hexactinellid species. Because our phylogeny (Fig. 2A) appears robust (Fig. 3), and we have many more sponge species analyzed, including multiple representative of hexactinellids, we estimated the divergence of the major silicean clades under the CIR model (the molecular clock model that best fit our data set), using branch lengths estimated under the CAT–GTR model of amino acid substitution (Fig. 4). Sensitivity analyses (Tables S1 and Table 3) suggested the results obtained using the CIR model, and a default soft bound relaxation level of 0.05% (see methods), are robust, and thus we present here only those results. The analyses identify five extant lineages with siliceous spicules as present in the Precambrian: the Silicea (Demospongiae + Hexactinellida) originating at 759 Ma, the Demospongiae at 699 Ma, Democlavia + Haplosclerida at 654 Ma, the Democlavia at 584 Ma, and the Haplosclerida at 586 Ma (Fig. 4, Table 2). We estimate the divergence between the Calcarea and Epitheliozoa (the origin of the Calcarea total group) at 754 Ma, and the origin of the Calcarea crown group at 487 Ma. Confidence intervals for these nodes are shown in Table 2 and Fig. 4.

Molecular divergence analyses conducted without the long-branched hexactinellids are only slightly different, with age estimates differing by approximately 5–10 Myr compared to analyses where they were included (Table 2, Table S1). Importantly, the estimated divergence between the spiculate haplosclerid and democlavid demosponges with the hexactinellids excluded is still Cyrogenian in age (661 Ma). Removing the prior on the root node and using hard bounds on calibration points causes ages to become slightly younger, but also still results in Cryogenian divergences for both Silicea and Haplosclerida + Democlavia (Table 3). Other sensitivity tests such as ignoring the maximum of 713 Ma for crown-group Demospongiae, relaxing the percentage of prior probability density of each calibration point lying outside the min–max interval, or assigning rates of evolution to the branches in our tree using either a correlated or an uncorrelated rate sampling strategy (Table S1, Table 3), only results in the estimates becoming deeper, and hence less consistent with the fossil record of siliceous spicules.

DISCUSSION

The suggestion by Brocks & Butterfield (2009) that the demosponge biomarkers reported from pre-Marinoan sediments in Oman (Love *et al.*, 2009) may not represent true

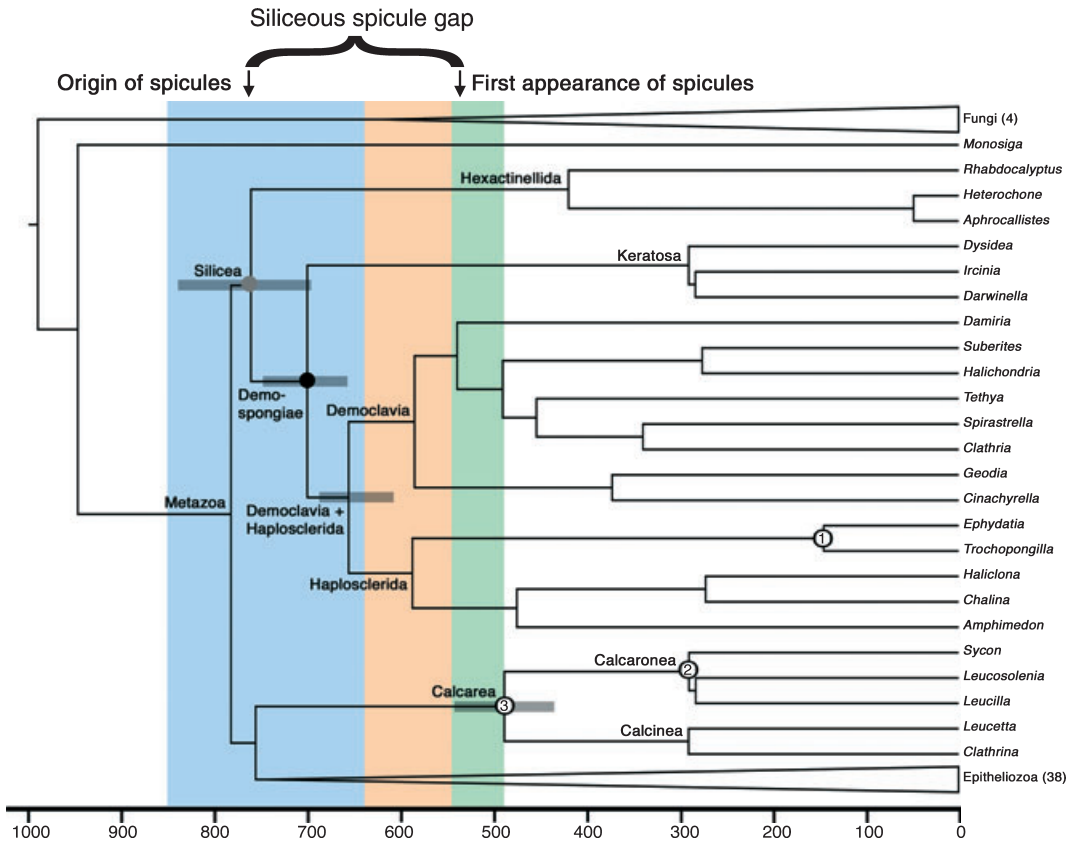

Fig. 4 Divergence times estimated under the CIR (rate autocorrelated) relaxed molecular clock model, the default soft bound relaxation level of 0.05, and the CAT–GTR amino acid substitution model. The phylogeny used to anchor the clock analysis is a pruned version of the phylogeny in Fig. 2 and includes only higher taxa whose phylogenetic position could be corroborated by the microRNA analysis (Fig. 3). According to this analysis, both the origin of metazoans and the diversification of siliceans occurred during the Cryogenian, with further diversification within the spicule-bearing demosponges occurring during the Ediacaran, long before the first appearance of spicules in the fossil record. This disparity highlights a 'spicule gap' between their presumed origin in the Cryogenian and their first appearances in the Early Cambrian. Note that our molecular divergence estimates are entirely in agreement with the biomarker record of demosponges (Love *et al.*, 2009) and with the Phanerozoic fossil record of sponges (nodes 1–3, see text).

Table 1 Number of miRNA reads from each taxon considered

	2014	2015	2016	2017	2018	2019	2020	2021	Parsed NR*
Aphrocallistes	0	0	0	0	0	743	0	0	57 288
Clathria	309	1947	1067	0	4	1192	878	1222	6,639
Dysidea	213	420	18	7	0	201	0	165	5270
Oscarella	0	0	0	0	0	0	0	0	11 564
Pleurobrachia	0	0	0	0	0	0	0	0	14 318
Suberites	1257	1028	1203	1	27	3735	444	6853	18 555
Sycon	0	0	1	0	0	0	0	0	16 044

*This number represents the number of parsed (i.e. reads between 17 and 25 nucleotides in length, and that meets minimum quality requirements, see Materials and methods) non-redundant reads.

multicellular sponges is based on the absence of a convincing Precambrian spicule record. This hypothesis requires that the sterol precursors were lost independently in multiple sponge and eumetazoan lineages, and that the demosponge total group is no older than the Cambrian (Fig. 1A). Alternatively, the absence of spicules could be accounted for if demosponges are paraphyletic with hexactinellids as the sister group of the spiculate haplosclerids and democlavids (Fig. 1B). The data presented here show that both possibilities are incorrect. Recent molecular phylogenetic studies (Philippe *et al.*, 2009; Sperling *et al.*, 2009) are corroborated by the application of microRNA-based phylogenetics, which place hexactinellids as the sister group of a monophyletic Demospongiae. Using this robust phylogeny, a molecular divergence estimate places the

Table 2 Estimated divergence times (±95%) for selected nodes in Fig. 4

	Soft bound relaxation level							
	Hexactinellida included				Hexactinellida excluded			
Taxon	0.05	0.1	0.25	0.5	0.05	0.1	0.25	0.5
Metazoa	781 (718, 838)	830 (748, 962)	956 (776, 1111)	1001 (863, 1127)	795 (730, 900)	857 (782, 982)	962 (829, 1133)	994 (870–1147)
Silicea	759 (698, 832)	803 (727, 935)	927 (758, 1057)	974 (827, 1103)	N/A	N/A	N/A	N/A
Hexactinellida	418 (291, 515)	427 (297, 525)	452 (305, 551)	463 (352, 551)	N/A	N/A	N/A	N/A
Demospongia	699 (660, 749)	731 (676, 846)	825 (704, 938)	867 (755, 976)	700 (670, 753)	746 (672, 874)	849 (737, 954)	892 (777, 1040)
Keratosa	289 (235, 343)	290 (253, 383)	277 (232, 360)	283 (239, 356)	282 (244, 324)	281 (244, 344)	281 (233, 358)	280 (225, 386)
Democlavia + Haplosclerida	654 (610, 689)	675 (607, 779)	742 (630, 859)	774 (672, 873)	661 (617, 723)	696 (631, 815)	753 (643, 856)	791 (674, 912)
Democlavia	584 (508, 643)	600 (517, 692)	645 (525, 750)	665 (580, 769)	594 (552, 638)	621 (548, 721)	654 (550, 754)	673 (548, 773)
Haplosclerida	586 (513, 634)	592 (521, 695)	625 (524, 744)	651 (537, 758)	587 (525, 632)	607 (524, 710)	631 (518, 757)	658 (550, 787)
Calcarea + Epitheliozoa	754 (695, 820)	800 (720, 918)	904 (758, 1072)	943 (814, 1073)	767 (688, 863)	815 (743, 913)	892 (766, 1052)	903 (790, 1021)
Calcarea	487 (433, 539)	487 (410, 578)	500 (402, 625)	496 (422, 596)	475 (427, 520)	474 (400, 532)	491 (416, 574)	492 (409, 593)

Divergence times estimated under the CIR (rate autocorrelated) relaxed molecular clock model and the CAT–GTR amino acid substitution model. Soft bound relaxation levels of 0.1, 0.25 and 0.5 are shown in addition to the default setting of 0.05, and analyses were conducted with and without the hexactinellids.

Table 3 Sensitivity analyses of molecular divergence estimates, showing estimated divergence times (±95%) for selected nodes in Fig. 4 using the CIR clock model with hard bounds on calibration points

	713 max included	713 max excluded
Metazoa	755 (693–809)	838 (747–952)
Silicea	740 (683–789)	823 (730–935)
Hexactinellida	440 (326–543)	467 (347–573)
Demospongia	692 (650–711)	773 (694–872)
Keratosa	288 (194–377)	316 (216–418)
Democlavia + Haplosclerida	648 (602–684)	718 (639–812)
Democlavia	586 (508–634)	647 (559–733)
Haplosclerida	572 (497–628)	625 (526–714)
Calcarea + Epitheliozoa	736 (683–789)	811 (720–920)
Calcarea	475 (382–564)	520 (422–610)

Analyses were conducted with the 713 Ma maximum for Demospongiae both included and excluded.

origin of siliceans deep within the Proterozoic, and suggests that the discordance between the spicule and biomarker record lies not with the organic geochemical record and its interpretation, but with the paleontological record of spicules.

The molecular clock analysis in this study indicates a gap of approximately 240 Myr between the origin of silicean spicules and their first unequivocal appearance in the fossil record (Fig. 4). What constitutes the first sponge spicules is contentious though – there are reports of siliceous spicules in the Ediacaran Duoshantuo Formation of China (Li *et al.*, 1998), the latest Ediacaran Kuibis Formation of Namibia (Reitner & Wörheide, 2002), and the latest Ediacaran Tsagaan Oloom Formation of Mongolia (Brasier *et al.*, 1997), but these reports are either single occurrences or their veracity has been questioned (Zhou *et al.*, 1998; Yin *et al.*, 2001). The first uncontested spicules appear in the Lower Cambrian at around 520 Ma (Bengtson *et al.*, 1990; Carrera & Botting, 2008). Thus, this 240-Myr gap could be reduced by accepting the Mongolian or Namibian spicules as real, but this would only reduce the gap by about 30 Myr, which does not resolve the fundamental discord between the molecular divergence estimates and the biomarker record versus the fossil record of siliceous spicules.

As noted in the results, our preferred estimates *minimize* the spicule gap between the estimated origin of siliceous spicules and their first appearance in the rock record (Fig. 4), but this gap could indeed be somewhat greater (Table S1, Table 3). Further, this analysis does not consider the homoscleromorph sponges, which also have siliceous spicules (Uriz *et al.*, 2003) and which are placed phylogenetically outside Demospongiae + Hexactinellida by both protein-coding genes (Philippe *et al.*, 2009; Sperling *et al.*, 2009) and microRNAs (this study). The spicule gap could therefore also potentially be lengthened if homoscleromorph spicules are homologous to demosponge spicules.

We see three potential solutions to this discordance: either the molecular clock estimates are inaccurate, silicean spicules are not homologous, or there is a taphonomic underpinning

to the first appearances of multiple lineages of sponge spicules in the Early Cambrian.

Inaccurate molecular divergence estimates

First, the divergence estimates reported here and elsewhere (Peterson *et al.*, 2008) could be wildly inaccurate. However, in addition to the general concordance between analyses of a similar data set and the bilaterian fossil record (Peterson *et al.*, 2008), our divergence estimates for other nodes within 'Porifera' appear to be accurate. These accuracy checks (Fig. 4) include: (i) an Early Cretaceous (145 Ma) divergence between the two freshwater sponges considered (*Ephydatia* + *Trochospongilla*) closely matches the Late Jurassic appearance of freshwater sponge gemmules (Finks *et al.*, 2004); (ii) the early Permian divergence between the included calcaroneans (which does not span the calcaronean crown group; Dohrmann *et al.*, 2006) post-dates the appearance of fossil calcaroneans in the early Carboniferous (Finks *et al.*, 2004); and (iii) the age estimates for the calcareous sponge crown group post-dates the appearance of calcareous sponges in the fossil record, all of which are likely stem-group calcareans (Finks *et al.*, 2004). Further, we stress the concordance between our estimate for the age of crown-group Demospongiae (699 Ma) and the stratigraphic appearance of the biomarkers between 635 and 713 Ma (Love *et al.*, 2009).

Modern molecular clock algorithms, such as those used in this study, are less sensitive to lineage-specific rate hetereogeneity in comparison to traditional, strict clock models. Accordingly, the inclusion of the long-branched hexactinellids necessary to date Silicea does not drive these deep dates (Table 2). When hexactinellids are excluded and only demosponges analyzed (whose branch lengths for this data set are similar to those of eumetazoans; Sperling *et al.*, 2009), a spicule gap of approximately 140 Myr still remains between the node Haplosclerida + Democlavia and the first appearance of spicules (Table 2). Such deep dates for the origin of demosponges are also not a novel result, as it is clear from qualitative analyses of branch lengths on the first protein-coding genes cloned from demosponges (Kruse *et al.*, 1998) that divergences within democlavids are roughly as deep as those for Bilateria. Therefore, it is unlikely that the estimates for crown-group Silicea, and the other Precambrian divergences within this clade, are so inaccurate as to eliminate the spicule gap.

Convergence of siliceous spicules

Second, it is possible that despite their obvious developmental and structural similarities (Leys, 2003; Uriz *et al.*, 2003; Kaluzhnaya *et al.*, 2007; Muller *et al.*, 2008a,b), the spicules of Silicea are not homologous, and spicules arose twice independently in this group. This would be consistent with the spicules of hexactinellids and demosponges having different organizations of the axial filament, and the fact that the hexactinellids and the spicule-bearing demosponges are separated phylogenetically by the aspiculate Keratosa (Figs 2A and 3). However, with the increased taxon sampling now available, our analyses suggest that several other divergences within the megasclere-bearing demosponges occurred prior to the first appearance of spicules in the fossil record, requiring independent evolution of identical siliceous spicules in sister clades for convergence to lie at the root of the spicule gap. For instance, we date the origin of the haplosclerids to 586 Ma, the origin of the democlavids to 584 Ma, and the origin of Haplosclerida + Democlavia to 655 Ma. In addition, there are most likely other spicule-bearing extant sponge lineages within both Demospongiae and Hexactinellida that diverged during the Precambrian. Although this data set has the widest poriferan taxonomic sampling of any protein-coding analysis to date, broader-scale ribosomal DNA phylogenies indicate there may be other lineages that, based on their phylogenetic relationships to the sponges studied in this paper, may also have Precambrian origins (Nichols, 2005; Redmond *et al.*, 2007; Dohrmann *et al.*, 2008). Hexactinellid and demosponge spicules are clearly similar, and both groups use the enzyme silicatein in spicule formation, an enzyme that is unknown outside of Silicea (Leys, 2003; Uriz *et al.*, 2003; Kaluzhnaya *et al.*, 2007; Muller *et al.*, 2008a,b). Given that Silicea is also monophyletic (Figs 2 and 3), siliceous megascleres are therefore likely homologous (Patterson, 1982), and not the products of convergent evolution in hexactinellids and in at least five different demosponge taxa at the base of the Cambrian.

Mega-taphonomic trends across the Precambrian–Cambrian boundary

The third possibility is a mega-taphonomic bias that caused the non-preservation of siliceous sponge spicules during the Cryogenian and Ediacaran. The primary support for this hypothesis comes from the fact that the molecular clock dates for organisms with calcareous skeletal elements, such as molluscs and echinoderms, closely match their first appearance in the fossil record (Peterson *et al.*, 2008). More pertinently, in contrast to the Silicea, our molecular divergence estimate for the Calcarea crown group at 488 Ma is not deeper than their known fossil record, but post-dates the first appearance of calcareous spicules (Finks *et al.*, 2004). Further, as mentioned above, our estimate for the divergence of the included calcaroneans post-dates the first appearance of the Calcaronea crown group in the fossil record, and the divergence between the freshwater demosponges matches the first appearance of gemmules in the fossil record (Finks *et al.*, 2004). Therefore, the discordance between the molecular divergence estimates and the fossil record does not extend to all skeletal organisms, or even to all sponges, but is a problem limited to the

Proterozoic fossil record of siliceous spicules alone. Indeed, consistent with a taphonomic hypothesis is the observation that there are no siliceous fossils in the Cryogenian, even in organisms that are inferred to have siliceous elements in life, but that are not preserved in the fossils themselves (Porter *et al.*, 2003).

Most experimental studies on the dissolution of biogenic silica in the modern ocean have been conducted on diatoms, and although sponge spicules will differ from diatom frustules in their taphonomy (Maldonado *et al.*, 2005), a great deal of the insights from these studies can be applied to the Proterozoic record. These studies have shown that the primary controls on the dissolution of biogenic silica are the amount of dissolved silica in solution, temperature, pH, salinity, the presence of organic and inorganic compounds that can be adsorbed by the silica, and the amount of reactive surface area exposed by skeletal elements (Van Cappellen & Qiu, 1997a,b). Although there are no direct measurements for dissolved silica levels in the Precambrian, both modeling studies (Siever, 1992) and sedimentological evidence based on the facies distribution of cherts through time (Maliva *et al.*, 1989) indicate that it may have been much higher prior to the base of the Cambrian. Thus, the inferred change in the amount of dissolved silica is opposite to that expected if a change in oceanic silicic acid concentration were to have caused the spicule gap. Likewise, temperature could have been involved, but the cool-water conditions of the Cryogenian (James *et al.*, 2005), which would inhibit dissolution, indicate that this is also unlikely.

In the modern ocean, the primary control on the solubility of biogenic silica is the amount of fine-grained siliciclastic detrital matter in the sediment (Van Cappellen & Qiu, 1997a,b; Dixit *et al.*, 2001; Cheng *et al.*, 2009). Incorporation of Al^{3+} in both synthetic silica (Iler, 1973) and cultured diatoms (Van Bennekom *et al.*, 1991) produces negatively charged aluminosilicate sites on the mineral surface that serve to reduce solubility by preventing the approach of the hydroxyl ions that catalyze dissolution (Iler, 1973). Studies of modern biosiliceous oozes demonstrate that diatom frustules continue to incorporate Al^{3+} into their crystal lattice after burial, to levels that are orders of magnitude above that achieved by the living organism (Dixit *et al.*, 2001). This is due to the early dissolution of clays, which release dissolved Al^{3+} to sedimentary pore waters, causing concentrations to rise orders of magnitude higher than in the open ocean. Thus, more clay in a sediment results in higher pore water dissolved Al^{3+}, higher Al/Si ratios in biogenic silica, and lower solubility of the biosiliceous particles.

Quantifying global trends in clay percentage through time is an imperfect art, but a large-scale study by Kennedy *et al.* (2006), who investigated the percentage of clays in the finest-grained units of three Neoproterozoic basins, found a steady increase in clay percentage from 775 to 525 Ma. Because of the lower dissolved Al^{3+} in pore waters of siliciclastic rocks during the Cryogenian and Ediacaran, there would necessarily be higher solubility of biogenic silica. Thus, we hypothesize that relatively clay-rich Cambrian sediments (perhaps operating synergistically with other factors) opened a taphonomic window for the preservation of siliceous sponge spicules.

CONCLUSIONS

Our data strongly suggest the presence of an approximately 240-Myr gap between the origin of siliceous sponge spicules and their first appearance in the fossil record. Although we hypothesize that this spicule gap was caused by a taphonomic bias due to the absence of Cryogenian and Ediacaran clay-rich sediments, we recognize that other factors might have played a role as well, including pH and independent increases to spicule size – these too will need to be tested.

More broadly, this example illustrates the power of molecular paleobiology (Peterson *et al.*, 2007) in providing an independent test of the geologic fossil record, and reveals that metazoan siliceous biomineralization has deep Precambrian roots, originating well before the Sturtian glaciation. And maybe even more interesting, our data suggest that siliceous spicules arose well before the origin of gut-bearing eumetazoans, supporting the suggestion that the original function of spicules in the Cryogenian was for structural support and that defense against modern predators represents an exaptation (Hill *et al.*, 2005; Jones *et al.*, 2005). In contrast, molecular clock ages for the calcareous sponges suggest that calcitic spicules were likely part of the broader Cambrian biomineralization event and their origin may share a similar cause. As with other mega-taphonomic trends around the Precambrian–Cambrian transition (Orr *et al.*, 2003; Porter, 2004) that would distort our view of this event if not taken into account, the sudden appearance of siliceous spicules near the base of the Cambrian likely represents the opening of a taphonomic window rather than an evolutionary first appearance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Sensitivity analyses of molecular divergence estimates, showing estimated divergence times (±95%) for selected nodes in Fig. 4

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