

Innovations in Silicon Transport Shaped the Rise of Biosilicification and Skeletal Evolution in Sponges

Laia Leria , Manuel Maldonado *

Department of Marine Ecology, Sponge Ecobiology and Biotechnology Group, Center for Advanced Studies of Blanes (CEAB, CSIC), Acceso Cala St. Francesc 14, Blanes, Girona 17300, Spain

*Corresponding author: E-mail: maldonado@ceab.csic.es.

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Abstract

Sponges are the only metazoans capable of making silica skeletons through incorporation of silicic acid (dSi) from seawater, which is polymerized using silicifying proteins. Uptake involves functional cooperation between aquaglyceroporin channels (gAQP) and arsenite efflux pumps (ArsB), a dSi transport system that, surprisingly, also functions in plants. Compared to plants, the silicon selectivity filter of sponge gAQPs is shown here to have replaced hydrophilic residues with hydrophobic ones, reducing water permeation during silicon transport. Phylogenetic analyses of 201 gAQP and 161 ArsB sequences reveal that these transporters, having prokaryotic origins, were already present in ancestral sponges, preceding the emergence of silicifying proteins and fossilized silica skeletons. Through Hexactinellida diversification, the functional interdependence of gAQP and ArsB transporters shaped a remarkable coevolution via synchronized gene duplications. This coevolution was disrupted in Demospongiae, because Heteroscleromorpha demosponges acquired, via horizontal gene transfer, a microbial gAQP that partially displaced ancestral gAQPs. This acquisition and that of an autapomorphic silicifying protein (silicatein) coincided with an exceptional diversification in Heteroscleromorpha. In contrast, sponge lineages that never developed silicifying proteins (Keratosa, Verongimorpha, Calcarea) or acquired them post-Cambrian (Homoscleromorpha, *Chondrilla*) lost gAQP genes while retaining ArsB homologs, implying selection against a passive dSi influx for sponges lacking dSi polymerization machinery. Thus, the ability to precipitate dSi—ie forming skeleton—likely arose as an adaptive response in early skeletal sponges to the damaging, high dSi concentrations of Precambrian oceans. The evolutionary history of dSi transporters and the fossil record support that such adaptation evolved independently four times within Porifera.

Keywords molecular evolution, silicification, silicon transport, early metazoans, Porifera, biomineralization

Introduction

Biomineralization is the biological production of inorganic compounds that often harden soft organic structures for skeletal purposes. Biosilicification is a prominent example, where silicifiers take up dissolved silicic acid (hereafter, dSi) from marine, continental, or soil waters and polymerize it into biogenic silica (hereafter, bSi) to make their skeletons. The continuous dSi consumption by silicifiers influences the cycling rates of silicon (Si) in major world's biomes, globally affecting the biogeochemical Si cycle across broad time and spatial scales (Maliva et al. 1989; Maldonado et al. 1999; Conley et al. 2017; Maldonado et al. 2019; Tréguer et al. 2021). The effects may ultimately reach the Earth's climate system through interactions between silicon and carbon biogeochemical cycles over geological timescales (Tréguer and Pondaven 2000; Isson et al. 2020)

Silicifying organisms include an array of unicellular groups (diatoms, chrysophyceans, silicoflagellates, radiolarians, thecamoebae, choanoflagellates, etc.), along with some multicellular

representatives such as macroalgae, plants, and sponges. The complex and only partially resolved evolutionary history of eukaryotic silicification suggests that different silicifiers independently developed distinct systems for polymerizing dSi. For instance, diatoms use long-chain polyamines (Sumper and Kröger 2004; Matsunaga et al. 2007; Durak et al. 2016), while sponges employ a variety of unrelated proteins across different lineages (Shimizu et al. 1998a; Shimizu et al. 2015; Shimizu et al. 2024) (Fig. 1a). Other marine silicifiers employ alternative, uncharacterized systems without recognizable homologs.

Sponges (phylum Porifera), an early-diverged lineage of Metazoa, are unique among animals for their ability to form bSi skeletons. The group comprises 9,781 species in four taxonomic classes (de Voogd et al. 2025). Three of them (Demospongiae, Hexactinellida, and Homosclerophorida) are characterized by all or some of their members developing bSi skeletons and collectively represent about 91.4% of extant species (Fig. 1a). A fourth class, Calcarea, constructs only calcareous skeletons.

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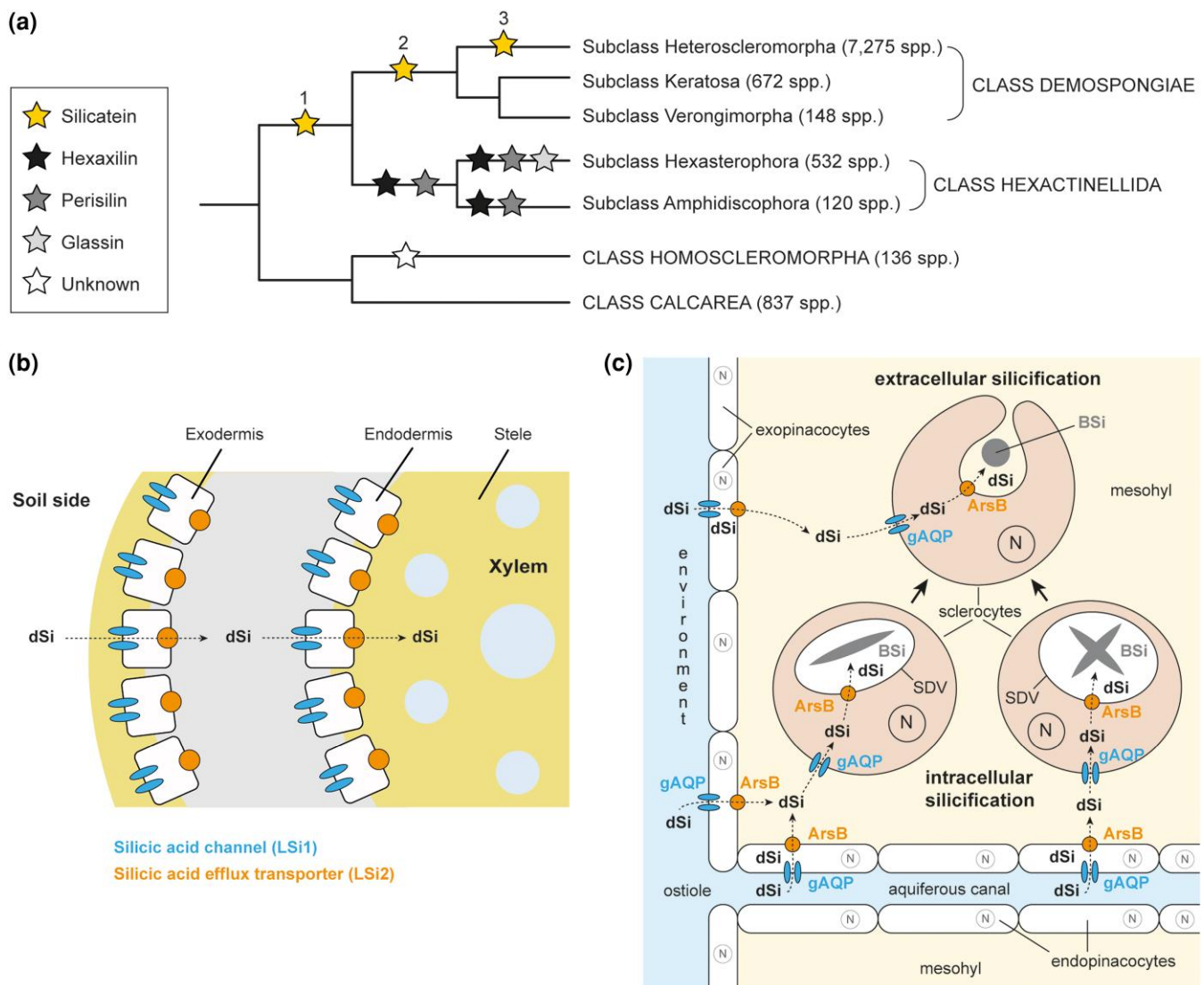


Figure 1 Summary phylogeny of Porifera and proposed mechanisms for dSi transport in plants and sponges. a) Mapping of the known and predicted silicifying proteins on the currently accepted phylogeny for the major lineages (taxonomic classes and subclasses) of the phylum Porifera. Silicatein, the first silicifying protein discovered in Porifera, has alternatively been proposed to be an acquisition in (1) the common ancestor of Hexactinellida and Demospongiae (Müller et al. 2007a), (2) in ancestral Demospongiae (Kozhemyako et al. 2010), and (3) in ancestral Heteroscleromorpha demosponges (Aguilar-Camacho et al. 2019, this study). Note that each hypothesis has, in turn, profound implications to interpret the absence of silicatein and siliceous skeletons in subclass Verongimorpha, which will be regarded a lost character under hypotheses 1 and 2, but a nonacquired character under hypothesis 3. (b) Schematic cross-section of a rice root (modified from Saitoh and Suga 2022), showing that Lsi1 channels are typically positioned in the plasmalemma of the distal side of the exodermal cells in the roots, facilitating dSi uptake from the soil solution into the cytoplasm of these cells. Subsequently, Lsi2 efflux pumps, positioned in the plasmalemma of the proximal side of the same cells, actively transport Si out of the cytoplasm into the apoplastic space of the cortex. From this location, Si is imported into the endodermal cells via another Lsi1 channel and subsequently transferred by another Lsi2 pump into the stele. From the stele, dSi is transported to the xylem via the transpirational stream (see Text S1). (c) Schematic cross-section of a demosponge summarizing dSi transport from the ambient water into the specialized silicifying cells (sclerocytes) where biogenic silica is produced through steps of intracellular and extracellular silicification (modified from Maldonado et al. 2020). As in plants, pairs of aquaglyceroporins (gAQPs) channels and active ArsB transporters are hypothesized to be positioned at the opposite poles of the various cell types (pinacocytes, sclerocytes, etc.) involved in the dSi transport pathway (see Text S1).

It is known that sponges in the class Hexactinellida use hexaxilin, glassin, and perisilin protein to polycondense dSi into skeletal silica (Shimizu et al. 2015, 2024), while sponges in the class Demospongiae do it using a variety of isoforms of the silicatein enzyme (Shimizu et al. 1998b; Müller et al. 2007b; Mohri et al. 2008). The silicifying proteins of sponges in the class Homoscleromorpha have not yet been identified. As all four silicifying proteins known in Porifera are phylogenetically unrelated

to each other, their collective message has been interpreted as an evidence that silicification evolved independently in the three siliceous sponge lineages (Shimizu et al. 2024). However, silicification involves more than just dSi polycondensation; it also requires a finely tuned system to transport dSi from the surrounding water to specific internal biological compartments where it will be polycondensed by silicifying proteins into skeletal silica. The evolutionary message in the elements of the

Porifera dSi transport system remains virtually unexplored (Maldonado et al. 2020).

Across the major silicifying eukaryotic lineages, dSi transport systems homology is hard to be traced, but some information is available (Marron et al. 2013). Genes encoding silicon transporters (SIT) were initially described in diatoms (Hildebrand et al. 1997; Thametrakoln and Hildebrand 2007; Hildebrand 2008) and homologs subsequently identified in other stramenopiles, such as chrysophyceans (Likhoshway et al. 2006) and additional members of the SAR supergroup (stramenopiles, alveolates, and rhizarians) (Durkin et al. 2016). Surprisingly, SIT-like genes were also identified in choanoflagellate protists, likely acquired via horizontal gene transfer from an haptophyte in the SAR supergroup (Marron et al. 2016). Before this discovery, choanoflagellates and sponges were expected to utilize phylogenetically related dSi transporters, since genomic evidence supports that choanoflagellates share a common ancestor with sponges and the rest of metazoans (King et al. 2008). However, it is now clear that choanoflagellates lack homologs of sponge silicifying proteins (such as silicatein, hexaxilin, glassin, perisilin) and that sponges lack the SIT-like transporters for dSi that characterize stramenopiles and choanoflagellates (King et al. 2008; Marron et al. 2016; Maldonado et al. 2020).

Complication was added to this evolutionary context, when sponges were proposed to transport dSi in a manner highly reminiscent of that described in phylogenetically distant organisms: terrestrial plants. This was discovered in individuals of the hexactinellid sponge *Vazella pourtalesii*, which, when exposed to elevated dSi concentrations, exhibited high dSi consumption rates while also upregulating two types of proteins involved in transmembrane transport, an aquaglyceroporin channel (gAQP3/9) and an arsenic efflux pump (ArsB) (Maldonado et al. 2020). These two antagonistic membrane transporters resulted to be distant homologs of another pair of antagonistic membrane transporters described from terrestrial plants, as Lsi1 channels and Lsi2 pumps (Ma et al. 2007, 2008; Coskun et al. 2021). Lsi1 is a passive aquaporin (AQP) channel relatively selective for Si (Mitani et al. 2008; Saitoh and Suga 2022; Zhou et al. 2022), while Lsi2 is a homolog of bacterial ArsB pumps but actively expels dSi (rather than arsenic) from the cytoplasm of root cells of land plants (Ma et al. 2007; Coskun et al. 2021; Zhou et al. 2022). In plants, these two antagonistic transporters are positioned in the plasmalemma of a same cell but at opposite cell poles, cooperating for dSi in soil water being translocated across successive cell layers of the roots into the xylem (Fig. 1b). The location and cooperative action mechanism of dSi transporters in plants strikingly resemble those proposed for sponges (Maldonado et al. 2020) (Fig. 1c, Text S1). The surprising discovery of homology in the dSi transport system between sponges and plants is to be added to the equally unexpected finding that sponge aquaglyceroporins are distant homologs of human aquaglyceroporins 3 and 9 (Maldonado et al. 2020). These human proteins still retain their ancestral “poriferan” functionality, facilitating the uptake of dSi in trace amounts by several cell types, including osteoblasts for bone formation (Garneau et al. 2015).

The intricate pattern of phylogenetic relationships outlined above suggests that dSi transporters have undergone a fascinating, dynamic, and yet poorly resolved evolutionary history, which has provided the primary motivation for this study. It remains unclear how the independent gene systems encoding

aquaglyceroporin and ArsB proteins diversified through Porifera evolution, how their functional interdependence has been preserved or modified, and what their fate has been in sponge lineages that lost or never acquired the capacity to silicify. The findings are expected to shed light on the evolutionary processes that enabled the emergence of silicification in sponges and the subsequent skeletal diversification of this ancestral metazoan lineage. Within a broader conceptual framework, the results also aim to refine our understanding of the origins and evolution of silicon-based biomineralization.

Results and discussion

Poriferan aquaglyceroporin channels for dSi transport

Evolutionary context

As detailed in Materials and methods, sponge aquaglyceroporins (gAQP) putatively transporting dSi were identified through blast searches in 40 publicly available genomes and transcriptomes of members of all four taxonomic classes of Porifera (Table S1). As a query, we used the amino acid (aa) sequence of the gAQP3/9 protein of the hexactinellid sponge *V. pourtalesii*, which resulted upregulated in individuals exhibiting increased dSi consumption rates during an experiment of dSi enrichment (Maldonado et al. 2020).

The study did not include other members of the aquaporin family known to occur in sponges, such as aquaporin 8 (Müller et al. 2011; Kenny et al. 2020; Santini et al. 2023). These channels are classical aquaporins, rather than aquaglyceroporins, and primarily transport water, although some may allow limited diffusion of solutes such as ammonium and oxygen peroxide. There is currently no evidence that their function involves dSi transport; if anything, they might facilitate the removal of water generated during silica polycondensation reactions (Müller et al. 2011; Wang and Müller 2015). Therefore, these classical AQPs are beyond the scope of this study, which focuses specifically on Si transporters.

To contextualize the evolution of sponge gAQPs, a comprehensive global scenario was generated by expanding the dataset up to 201 sequences (File S1), incorporating putative protein homologs retrieved through blast searches across all domains of life (Tables S2 to S4). We included bacterial and archaeal aquaporins Z (AqpZ) as likely ancestors of eukaryotic classical, 8-type, and unorthodox aquaporins, while bacterial and archaeal glycerol facilitator channels (GlpF) were included as putative ancestors of aquaglyceroporins (Finn et al. 2014).

The ML and BI phylogenetic analyses of our AQP dataset retrieved the major types of aquaporins (sensu Soto et al. 2012; Abascal et al. 2014; Li and Wang 2017) as monophyletic groups (Fig. 2, Fig. S1). Nodulin26 and Lsi1 transporters of plants were positioned, along with other non-aquaglyceroporins, outside the gAQP superclade. Within the gAQP superclade, sponge sequences unexpectedly formed two distinct, distant clades. Clade 1 (expanded in Fig. 3) contained gAQP sequences of demosponges exclusively within the subclass Heteroscleromorpha, but not of subclasses Keratosa and Verongimorpha. Clade 2 (expanded in Figs. 4 and 5) included sequences of the classes Demospongiae and Hexactinellida, each conforming to a highly supported monophyletic group. A partial sequence of

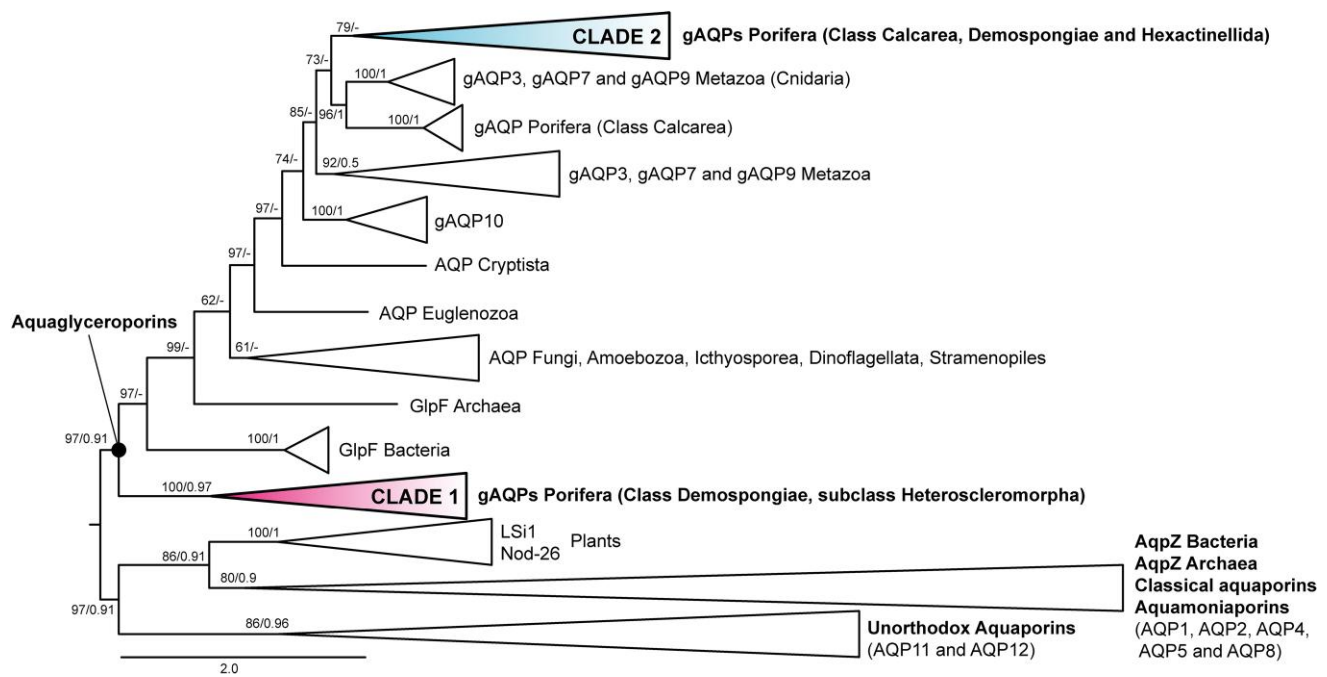


Figure 2 Phylogenetic context of sponge aquaglyceroporins within the superfamily of major intrinsic proteins. Poriferan aquaglyceroporins are distributed in two main, distant clades, namely, Clade 1 (highlighted in pink) and Clade 2 (highlighted in blue). The phylogeny was inferred using maximum likelihood via IQ-TREE software. At nodes, values 1 to 100 indicate bootstrap support (bs), while values 0 to 1 are posterior probabilities (pp) obtained through Bayesian inference (bs/pp). See Fig. S1 for the Bayesian phylogeny and File S1 for sequences. Scale bar represents the number of amino acid substitutions per site.

Leucosolenia complicata (class Calcarea, Porifera) slipped between the two sponge classes, as an artifactual, weakly supported sister branch to Hexactinellida (see Text S2). Unlike this outlying controversial sequence, the other Calcarea gAQP sequences formed a sister-group relationship with non-silicifying, non-sponge metazoans. Notably, the three members of the sponge class *Homoscleromorpha* considered in the dataset lacked homologs to the sponge gAQP sequences from Clade 1 and Clade 2, an important absence with significant implications for reconstructing the evolutionary history of silicification and skeletal diversification in siliceous Porifera.

Sponge gAQP genes in Clade 2 appear to have evolved from ancestral gAQP forms inherited from the poriferan ancestor (Fig. 2). In contrast, sponge gAQP sequences in Clade 1 form a sister-group relationship with bacterial GlpFs and represent the earliest diverging lineage of all aquaglyceroporins (Fig. 2). The unexpected basal position of sponge gAQP Clade 1, coupled with its weak relationship to other sponge gAQP clades and its closer relationship to bacterial gAQP, suggest that ancestral *Heteroscleromorpha* demosponges acquired these specific proteins via horizontal gene transfer (HGT) from bacteria. This subject will be explored in a dedicated subsequent section, as these HGT-acquired gAQP channels appear to have been crucial in the skeletal evolution of *Demospongiae*.

Demosponge gAQP proteins in Clade 1: horizontal gene transfer

To further investigate the origin and evolutionary history of sponge gAQP proteins in Clade 1, we selected five complete sequences representative of this clade and conducted additional blast searches in the NCBI database. The best-scoring hits of all five searches corresponded to bacterial aquaglyceroporins,

primarily from the genera *Chloroflexota* and *Dehalococcoidia*, within the phylum *Chloroflexi* (Table S5). These are filamentous bacteria common in marine sediment microhabitats (Wasmund et al. 2014) but also identified as sponge symbionts (Hentschel et al. 2012). While the presence of these symbionts in sponges might suggest that gAQP genes belong to the symbionts rather than to the heteroscleromorph sponges, several lines of evidence strongly support the sponge origin of these sequences. First, the phylogenetic analysis incorporating blast hits of sponge sequences, and having bacterial GlpFs as an outgroup, revealed that most sponge gAQP sequences ($N = 43$) formed a well-supported monophyletic group, distinct from bacterial *Chloroflexi* sequences. Furthermore, the internal structure of this clade aligns with the currently accepted phylogeny of *Heteroscleromorpha* (Fig. 3). In contrast, only four sequences of *Geodia barretti* and one of *Xestospongia testudinaria* clustered among bacterial sequences. Second, multiple independent duplications of this gene occurred within species such as *G. barretti* and *Amphimedon queenslandica* (with at least two genes) or *Theonella swinhoei* and *Petrosia ficiformis* (with three genes). In freshwater sponges (order Spongillida), at least two duplication events were shared by all species, with additional independent duplications observed in certain species. Across the species, most duplicated genes occurred in the same chromosome, ruling out contamination from microbial sequences. Thus, these traits support that sponge gAQP proteins in Clade 1 were likely acquired through horizontal gene transfer (HGT) and probably from a bacterial *Chloroflexi* symbiont into the ancestral *Heteroscleromorpha*. Henceforth, these aquaglyceroporins will be referred to as “gAQP_Het_Chloroflexi.”

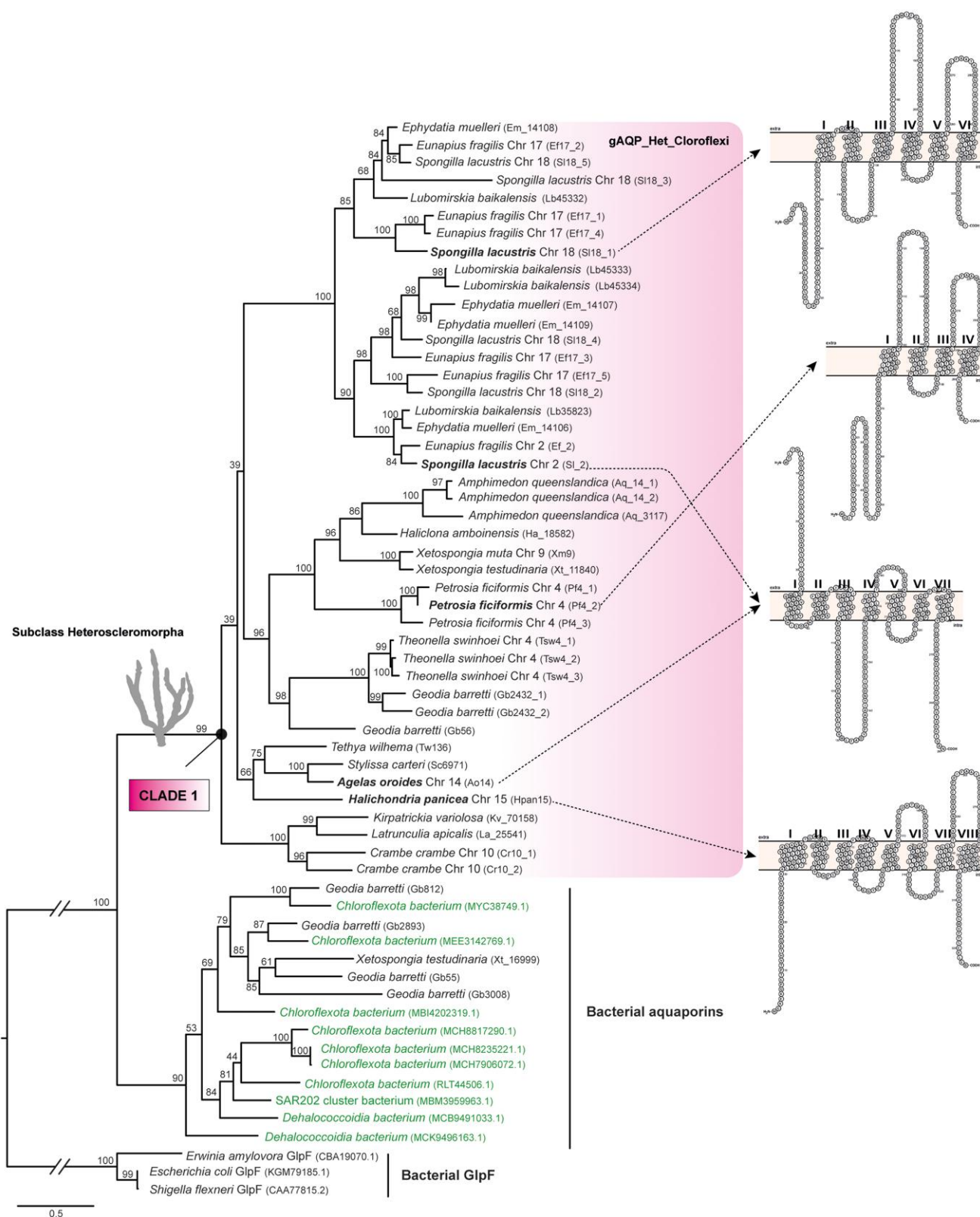


Figure 3 Phylogenetic relationships of demosponge aquaglyceroporins from Clade 1 and their secondary structure. The phylogeny was inferred using maximum likelihood via IQ-TREE software. Values at nodes correspond to the bootstrap support. Labels in brackets after the OTUS name of taxa are sequence codes in the alignment. Sequences colored in green are bacterial aquaporins of the phylum Chloroflexi, which together with sponge sequences in Clade 1, show a sister-group relationship with bacterial glycerol uptake facilitator proteins (GlpF). Secondary structures were inferred for a representative of the different clades (species names in bold) using Protter software. Roman numerals in the secondary structures identify different transmembrane protein domains. Loops above and below the membrane correspond to extracellular and cytosolic regions, respectively. See [File S1](#) for sequences. Scale bar represents the number of amino acid substitutions per site.

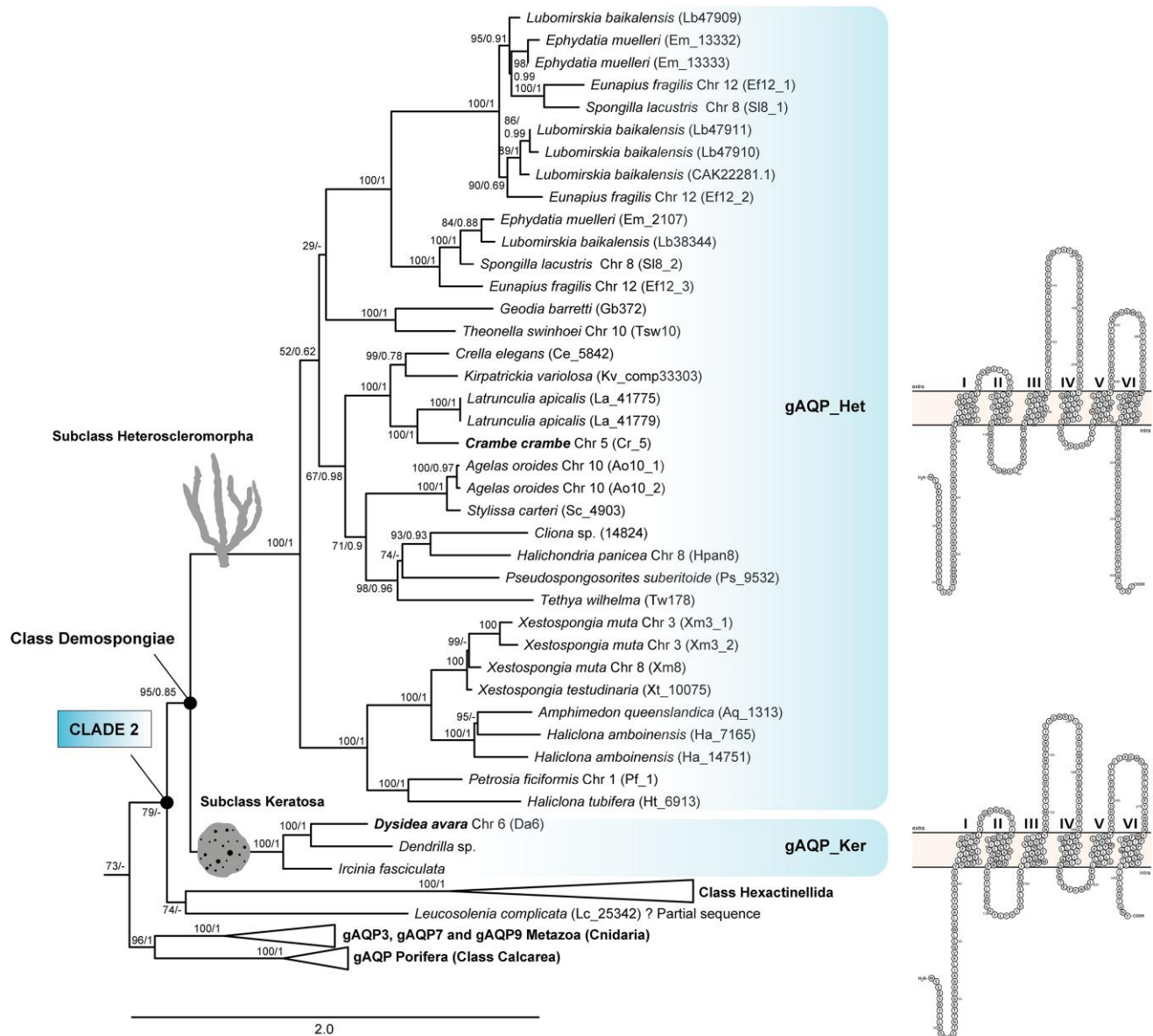


Figure 4 Phylogenetic relationships of dem sponge aquaglyceroporins in Clade 2 and secondary structure of clade representatives. The phylogeny was inferred with maximum likelihood via IQ-TREE software and showed identical topology when inferred with Bayesian methods. Numbers at nodes correspond to values of bootstrap support and posterior probability (bs/pp), respectively. Labels in brackets after the name of taxa correspond to the sequence codes in the alignment. Secondary structure was inferred using Protter software for a representative gAQP member of each clade (species names in bold). Labels in Roman numerals in the secondary structures identify different transmembrane protein domains. Loops above and below the membrane correspond to extracellular and cytosolic regions, respectively. See [File S1](#) for sequences. Scale bar represents the number of amino acid substitutions per site.

In line with the suggestion of an HGT origin for the sponge gAQPs of Clade 1, it was recently reported that freshwater sponge *Ephydatia muelleri* contains nine paralogs of bacterial *GlpF* genes used for glycerol transport, five of which are located on the same scaffold (Kenny et al. 2020). This finding challenged the traditional view that *GlpFs* occur only in bacteria and plants but not in animal genomes (Gustavsson et al. 2005) and led to the hypothesis that their presence in sponges, as in plants, may have arisen via horizontal gene transfer (Kenny et al. 2020). Although Kenny et al. associated these sponge *GlpF* channels with glycerol transport rather than dSi transport, our analyses indicate their *GlpF* channels correspond indeed to “our” gAQP_Het_Chloroflexi genes. Therefore, both studies have independently supported the possibility of an HGT for

Clade 1 of sponge gAQPs. Since phylogenetic and structural analyses (see subsequent results) indicate that these sponge gAQPs have diverged substantially from their ancestral bacterial *GlpFs* and now form a distinct subclade within the broader gAQPs clade, we refer to them as aquaglyceroporins rather than *GlpFs*.

Demosponge gAQP proteins in Clade 2: erratic diversification

Currently, the accepted phylogenetic relationships recognized between the three subclasses of Demospongiae are ((Verongimorpha, Keratosa) Heteroscleromorpha) (Morrow and

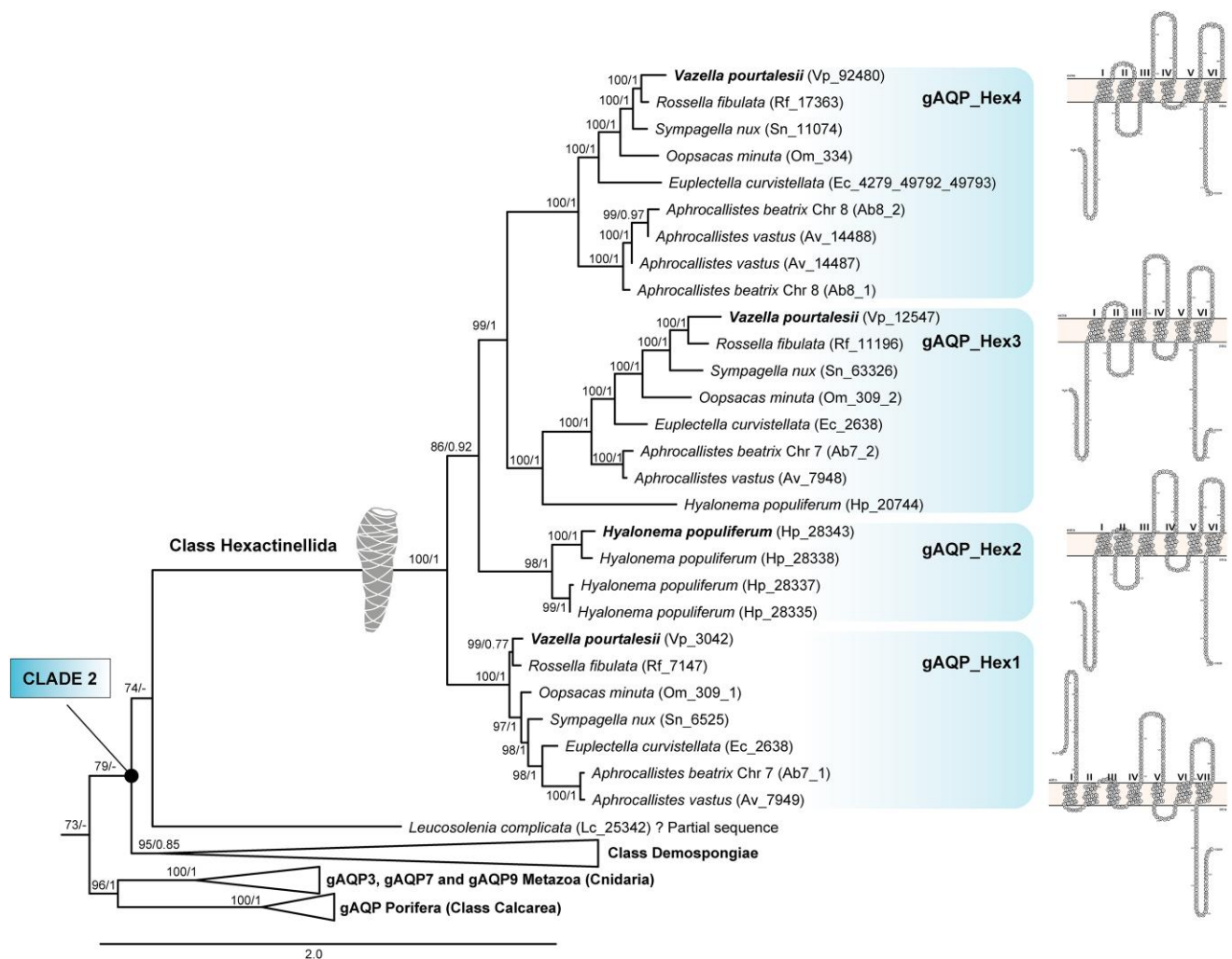


Figure 5 Phylogenetic relationships of hexactinellid aquaglyceroporins and secondary structure of clade representatives. The phylogeny was inferred with maximum likelihood via IQ-TREE software and showed identical topology when inferred with Bayesian methods. Numbers at nodes correspond to values of bootstrap support and posterior probability (bs/pp), respectively. Labels in brackets after the name of taxa are sequence codes in the alignment. Secondary structures were inferred using Protter software for a representative gAQP member of each hexactinellid clade (species names in bold). Roman numerals in the secondary structures identify different transmembrane protein domains. Loops above and below the membrane correspond to extracellular and cytosolic regions, respectively. See [File S1](#) for sequences. Scale bar represents the number of amino acid substitutions per site.

Cárdenas 2015; Lavrov et al. 2023) (Fig. 1a). Demosponge gAQP proteins in Clade 2 formed a monophyletic group with distinct, well-supported internal subgroups that corresponded to the recognized subclasses (Fig. 4). However, since all four Verongimorpha representatives in the dataset lacked detectable homologs of these gAQP channels, the subclass is not present in the phylogenetic tree, leading to gAQP sequences of Keratosa (gAQP_Ker) and Heteroscleromorpha (gAQP_Het) to form a sister-group relationship (Fig. 4).

Heteroscleromorpha gAQP sequences were widely diversified. Diversification followed no consistent pattern in the number of gAQP proteins per species (species typically represented taxonomic orders in the phylogenetic tree), which varied nonsystematically from 1 to 4. A major exception to this uneven diversification pattern was freshwater sponges (order Spongillida), which had two to five distinct gAQP proteins per species (Fig. 4), likely resulting from duplications in the ancestral Spongillida. The global heterogeneity characterizing the “erratic”

diversification pattern of Heteroscleromorpha gAQP proteins in Clade 2 contrasts with the highly ordered pattern observed in Hexactinellida (see the following section).

The persistence of ancestral gAQP genes (gAQP_Ker) for dSi transport in certain species of the subclass Keratosa was unexpected, since no member of this demosponge subclass is capable of producing a siliceous skeleton. In contrast, these ancestral gAQP genes have been completely lost in Verongimorpha, the sister subclass of Keratosa (Fig. 1a), which is likewise largely incapable of silica production. Within this evolutionary context, it is striking that a single Verongimorpha genus, *Chondrilla* (16 spp.), is able to produce a structurally simple skeleton composed of thousands of small (20 to 100 μ m in diameter), siliceous, star-like spicules known as spherasters. Such a capability must have arisen through the “de novo” evolution of proteins enabling both dSi intake and its subsequent polymerization (Text S3), since our genomic analyses confirmed that *Chondrilla nucula* lacks both gAQP_Het_Chloroflexi genes of Clade 1 and gAQP genes of

Clade 2 responsible for dSi transport in other sponge lineages, as well as genes encoding the silicifying proteins known from heteroscleromorph demosponges and Hexactinellida.

The idea of two independent origins for the emergence of silicification in Demospongiae, once in Heteroscleromorpha and once in the Verongimorpha genus *Chondrilla*, also agrees with the fossil record. The oldest unequivocal Heteroscleromorpha spicules date from the Lower Cambrian, some 515 mya (Botting et al. 2015), while the earliest “chondrillid” spherasters are about 100 million years younger, based on the description of *Syringostroma* Nicholson, 1875, from the Middle Devonian (419 to 359 mya). Subsequent “chondrillid” fossils were described as *Chondrochaetetes longitubus* Reitner, 1991, from the Carboniferous (359 to 299 mya) and *Calcichondrilla incrustans* Reitner, 1991, from the Early Cretaceous (145 to 100 mya). Importantly, in all three “chondrillid-like” fossils, the siliceous skeleton of spherasters co-existed with a basal calcareous skeleton. This suggests that the second emergence of silicification within Demospongiae led to the development of sclerosponge-like skeletons in the class Verongimorpha (Text S4). How and why the *Chondrilla*-like lineage lost its ancestral calcareous skeleton remain unknown, but this observation opens up new scenarios for investigating the evolution of demosponge lineages that combined calcareous and siliceous skeletons, informally referred to as “sclerosponges” or “coralline sponges” (Hartman and Goreau 1970; Lang et al. 1975; Willenz and Hartman 1989). It cannot be ruled out that the relatively late emergence of skeletonization through silicification was particularly advantageous for this small sponge lineage and may have displaced the pre-existing molecular and cellular mechanisms responsible for forming an ancestral calcareous skeleton.

Genome explorations also revealed that the sister classes Homoscleromorpha and Calcarea (Fig. 1a) both lack ancestral gAQPs. Under the principle of maximum parsimony, this absence is best explained by a single gene loss in their common ancestor rather than by two independent losses, one in each class. The loss of these gAQPs in both sponge classes may have somehow prevented or hindered the emergence of silicification in these lineages. Indeed, calcareous sponges never evolved the ability to silicify (what might have facilitated the emergence of calcification). Currently, calcareous sponges represent only about 8.6% of extant sponge species and have, on average, a smaller body size than demosponges and hexactinellids. This generalized trait could derive, among other factors, from architectural limitations of their calcareous skeletons. Likewise, the absence of ancestral gAQPs in the Homoscleromorpha lineage could be related to the fact that, according to the fossil record, this lineage acquired the ability to silicify somewhere between the Carboniferous (Reid 2004) and the Upper Jurassic (Wiedenmayer 1994), that is, hundreds of million years later than Hexactinellida (Chang et al. 2017) and Demospongiae (Botting et al. 2015), which did it in the early Cambrian.

Hexactinellid gAQP proteins in Clade 2: ancestral systematic duplications

In contrast to the irregular diversification pattern observed in demosponge gAQP proteins, hexactinellid gAQP proteins in Clade 2 exhibited a highly organized diversification, forming four well-supported groups (Fig. 5): (i) gAQP_Hex1 group, likely ancestral to the others, consisted of all species from the subclass

Hexasterophora; (ii) gAQP-Hex_2 contained only sequences of *Hyalonema populiferum*, the sole representative from the subclass Amphidiscophora with publicly available data; (iii) gAQP_Hex3 group included all species from both Hexactinellida subclasses; and (iv) gAQP_Hex4 group comprised all species from only the subclass Hexasterophora. Notably, all four clades contained a single gene per species, except *Aphrocallistes* species (ie *A. beatrix* and *A. vastus*), which showed a duplication of the gAQP_Hex4 gene. Such a diversification pattern likely arose from two gAQP duplication events in the ancestors of the class, followed by one gene loss in the subclass Amphidiscophora (Text S5) and an additional duplication in the genus *Aphrocallistes*. This evolutionary process resulted in all hexactinellids of the order Lyssacinosa having three gAQP proteins, all Scepstrulophora four, and the sole Amphidiscophora in the data set five (Table S6). Exactly these same gene numbers in these same taxa are obtained when analyzing the diversification of ArsB transporters (as will be shown in a subsequent section), uncovering a remarkable example of parallel evolution of two independent gene families, whose proteins must work with functional interdependence for an efficient transcellular translocation of dSi.

Sponge ArsB pumps for dSi transport

Evolutionary context

Putative sponge ArsB transporters for dSi were identified through blast searches in 40 publicly available genomes and transcriptomes of representatives in all four taxonomic classes of Porifera (see Materials and methods). As a query, we used the aa sequence of the ArsB1 protein of the hexactinellid sponge *V. pourtalesii*, which resulted upregulated in individuals that exhibited increased dSi consumption rates during an experiment of dSi enrichment (Maldonado et al. 2020). To contextualize the evolution of the poriferan ArsB, a comprehensive scenario was generated by expanding the dataset up to 161 sequences (File S2), with protein homologs searched by blast in databases including all domains of life (Table S7 to S9). Bacterial ArsB pumps were included as putative ancestors.

The phylogenetic analyses using both BI and ML inference resulted in similar tree topologies (Fig. 6a, Fig. S2). Bacterial ArsB sequences represented the first diverging lineage in the phylogeny. Sponge ArsB sequences formed a well-supported monophyletic group, sister to different lineages of both non-silicifying and silicifying eukaryotes (Text S6). The sponge ArsB clade consisted of three highly supported monophyletic groups, each corresponding to a taxonomic class: Hexactinellida, Homoscleromorpha, and Demospongiae. Importantly, no ArsB homologs were found in sponges of the class Calcarea (Fig. 6a), an absence of character that must be interpreted as a loss of the ancestral ArsB pumps.

Hexactinellid ArsB proteins: function-driven coevolution

Hexactinellid ArsB sequences made four well-supported groups, which exhibited taxonomic coherence within and between them (Fig. 6b), rigorously paralleling the diversification pattern shown by hexactinellid gAQP sequences (Fig. 7): (i) clade ArsB_Hex1 contained species from only the subclass Hexasterophora; (ii) clade ArsB_Hex2 contained only sequences of *H. populiferum*, the sole representative from the subclass Amphidiscophora; (iii) clade

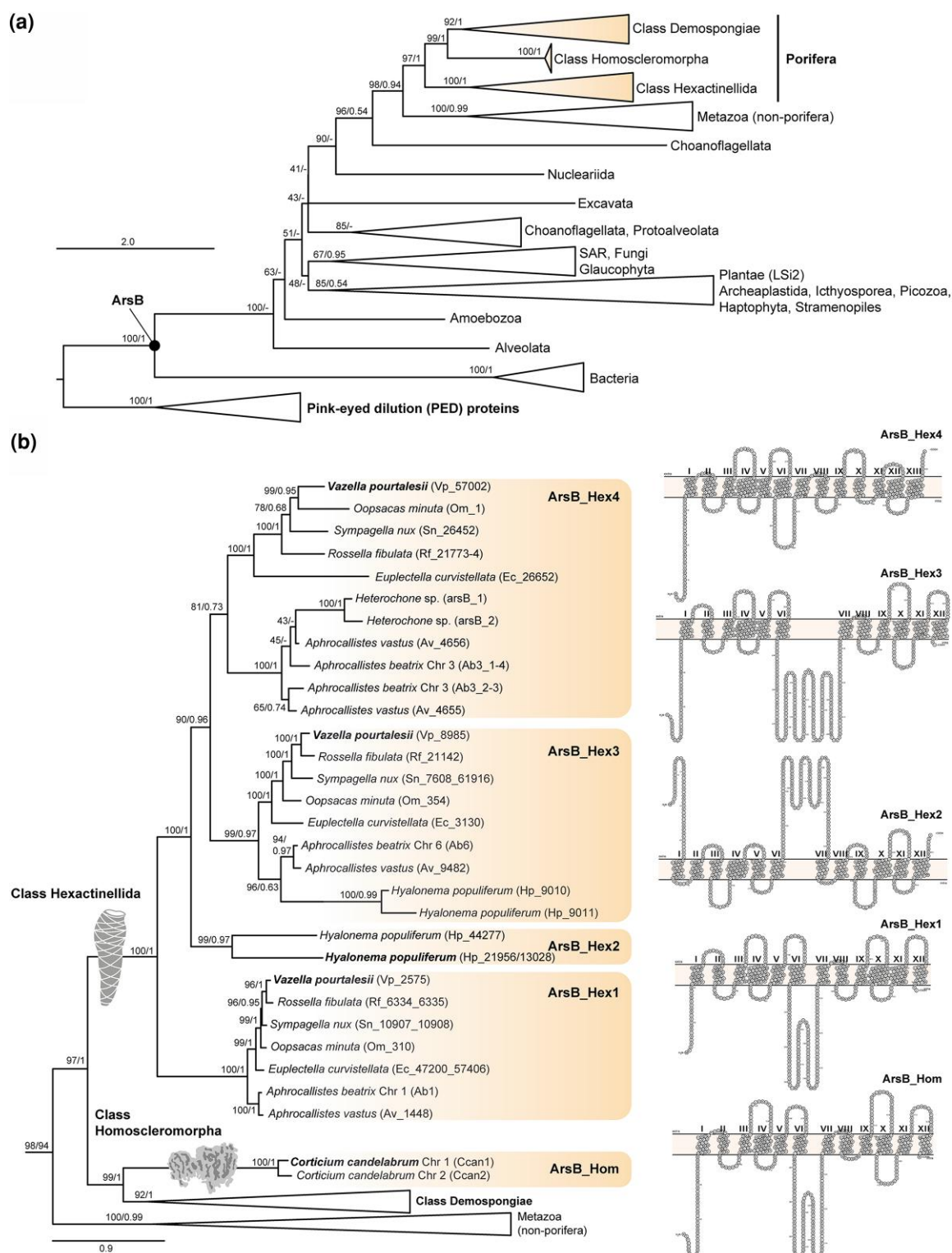


Figure 6 Phylogenetic context of sponge ArsB transporters and relationships within classes Hexactinellida and Homoscleromorpha. Both phylogenies were inferred with maximum likelihood via IQ-TREE software and showed identical topology when inferred with Bayesian methods. Labels in brackets after the name of taxa are sequence codes in the alignment. Numbers at nodes correspond to bootstrap support and posterior probability (bs/pp), respectively. The scale bar represents the number of amino acid substitutions per site. a) Sponge ArsB transporters are present in the three siliceous sponge classes, but not in Calcarea, and make a monophyletic group in the global context of the analysis (clades highlighted in orange). Pink-eyed dilution (PED) proteins were used as an outgroup. b) Phylogenetic relationships of ArsB transporters, displaying the monophyletic clades found for classes Hexactinellida and Homoscleromorpha. Secondary structure has been inferred for representatives of the main phylogenetic clades (species names in bold) using Protter software. Labels in Roman numerals in the secondary structures identify different transmembrane protein domains. Loops above and below the membrane correspond to extracellular and cytosolic regions, respectively. See [File S2](#) for sequences.

ArsB_Hex3 comprised all species from both subclasses; and (iv) clade ArsB_Hex4 contained all species from subclass Hexasterophora. Again, following a remarkable parallelism with the diversification pattern of hexactinellid gAQP, all these ArsB clades were represented by a single gene per species, except in *A. beatrix* and *A. vastus*, both of which showed a duplication of ArsB_Hex4 (Figs. 6b and 7). As for hexactinellid gAQP proteins, the diversification of the hexactinellid ArsB proteins appears to have been based on two events of duplication in the ancestors of the class, followed by one gene loss in the subclass Amphidiscophora and an additional duplication in the genus *Aphrocallistes*. Such a coevolution has led to species in the order Lyssacosinosa having exactly three ArsB and three gAQP, and species in the order Sceptulophora four of each (Fig. 7). The only member of the subclass Amphidiscophora, *H. populiferum*, was predicted to have five of each, but only four ArsB could be identified, a mismatch likely resulting from the moderate quality of this genome (Text S5).

The striking coevolution pattern observed between gAQP and ArsB proteins likely stems from the necessity for gAQP channels and ArsB pumps to function as coupled pairs for efficient transcellular dSi translocation (Fig. 1c). This functional interdependence appears to have acted as a strong selective force, precisely shaping the coevolution of gAQP channels and ArsB pumps throughout the skeletal diversification of the class Hexactinellida (Fig. 7).

Venturing into speculative territory, we hypothesize that the ancestral gAQP_Hex-ArsB_Hex pair of transporters was likely located in the membrane of epithelial cells, which were not specialized in silicification (Fig. 1a) but nevertheless regulated the passage of ambient dSi into the mesohyl. As some mesohyl cells became specialized in silicification (ie syncytial sclerocytes) and further diversified, this ancestral gAQP-ArsB system likely gave rise to the remaining variants, thereby promoting skeletal evolution in both hexactinellid subclasses. If this hypothesis is correct, the diversification of the gAQP-ArsB system should parallel the diversification of the silicifying cell lineages, leading to the evolution of multiple spicule types (hexactins, monaxons, microscleres, etc.), as well as the emergence of extracellular silicification, which enables the formation of very large spicules beyond the constraints of intracellular synthesis. Thus, given the structured coevolution of the gAQP and ArsB gene families through the diversification of the class Hexactinellida, these gene families may serve as additional suitable molecular markers to help clarify the evolutionary history and phylogenetic relationships within this sponge class.

Demosponge ArsB proteins: HGT-disrupted coevolution

Demosponge ArsB sequences exhibited a relatively structured diversification pattern through duplications (Fig. 8). However, unlike in Hexactinellida, this pattern was not paralleled in the diversification of their gAQP counterparts (Fig. 5). Demosponge ArsB proteins formed two major groups (Fig. 8). One group included the subclasses Keratosa (ArsB_Ker) and Verongimorpha (ArsB_Ver) as sister, well-supported, monophyletic clades. These two subclasses share the lack of a siliceous skeleton (with the exception of the verongimorph genus *Chondrilla*). The other group corresponded to the subclass

Heteroscleromorpha, containing ArsB sequences grouped into three clusters (Fig. 8). Clade ArsB_Het1 and Clade ArsB_Het3 included sequences of the orders Agelasida, Poecilosclerida, Scopalinida, Spongillida, Tethyida, and Tetractinellida. They differed in clade ArsB_Het3 additionally including sequences of Suberitida and Haplosclerida (Fig. 8). For practical reasons, clade ArsB_Het2 was selected with a paraphyletic structure, including a well-supported monophyletic clade of Haplosclerida along with another monophyletic group represented by the orders Tetractinellida, Tethyida, Suberitida, and Poecilosclerida. From a strictly phylogenetic perspective, selecting for a paraphyletic structure may appear an odd decision. However, the high support of these clades suggests the possibility of a functional divergence (ie specialization in two distinct sclerocyte lineages) between the two ArsB clusters.

In summary, all orders of the subclass Heteroscleromorpha showed at least two highly differentiated types of ArsB transporters, which became three in the case of Poecilosclerida, Tethyida, and Tetractinellida. Notably, unlike in Hexactinellida, the number of sequences within an ArsB clade not only varied between species but also seldom matched the number of gAQP sequences in Clade 2 (Table S6).

The question now is why the remarkable coevolution between ArsB pumps and gAQP channels observed in Hexactinellida (Fig. 7) did not occur in Demospongiae. Both Heteroscleromorpha demosponges and Hexactinellida require functional cooperation between gAQP channels and ArsB pumps to achieve translocation of dSi across each cellular barrier (Fig. 1c). The need of such a coordinated action likely exerted strong selective pressure and, through systematic gene duplications, drove a remarkable coevolution of the ancestral gAQP channels and ArsB pumps during the skeletal diversification of the class Hexactinellida (Fig. 7). However, coevolution in Heteroscleromorpha demosponges appears minimal, mostly because the pattern of ArsB duplications (which are in line with the pattern previously described in Hexactinellida) is not mirrored by the gAQP genes.

The most likely cause is the horizontal transfer of gAQP_Het_Chloroflexi gene to the Heteroscleromorpha ancestor: The gAQP channels, acquired via HGT, began coupling with ArsB pumps for dSi transport, largely (but not completely) displacing ancestral gAQP channels from this role. Replacement could derive from a superior functional performance in dSi transport, since preliminary research indicates that out of the three gAQP channels identified in the poecilosclerid demosponge *Crambe crambe* (Table S6), one of the two HGT-acquired channels is about twice faster in dSi transport than the ancestral sponge gAQP (Maldonado et al., unpublished). Improved capabilities for dSi transport are also in line with our discovery that the aa sequence of the Si selectivity filter of the HGT-acquired gAQP channels has evolved to maximize Si transport with minimum water permeation compared to that of ancestral gAQPs, which have a Si selectivity filter more similar to that of plant LSi transporters (see section on “Structural comparison of dSi transporters”). While the HGT acquisition of these microbial gAQP channels may have facilitated dSi uptake in this demosponge subclass, it is also likely that it disrupted the original pairing between ancestral sponge gAQP channels and ArsB pumps, with more efficient HGT-acquired gAQP channels displacing ancestral gAQP from the coupling with ArsB pumps. Supporting evidence

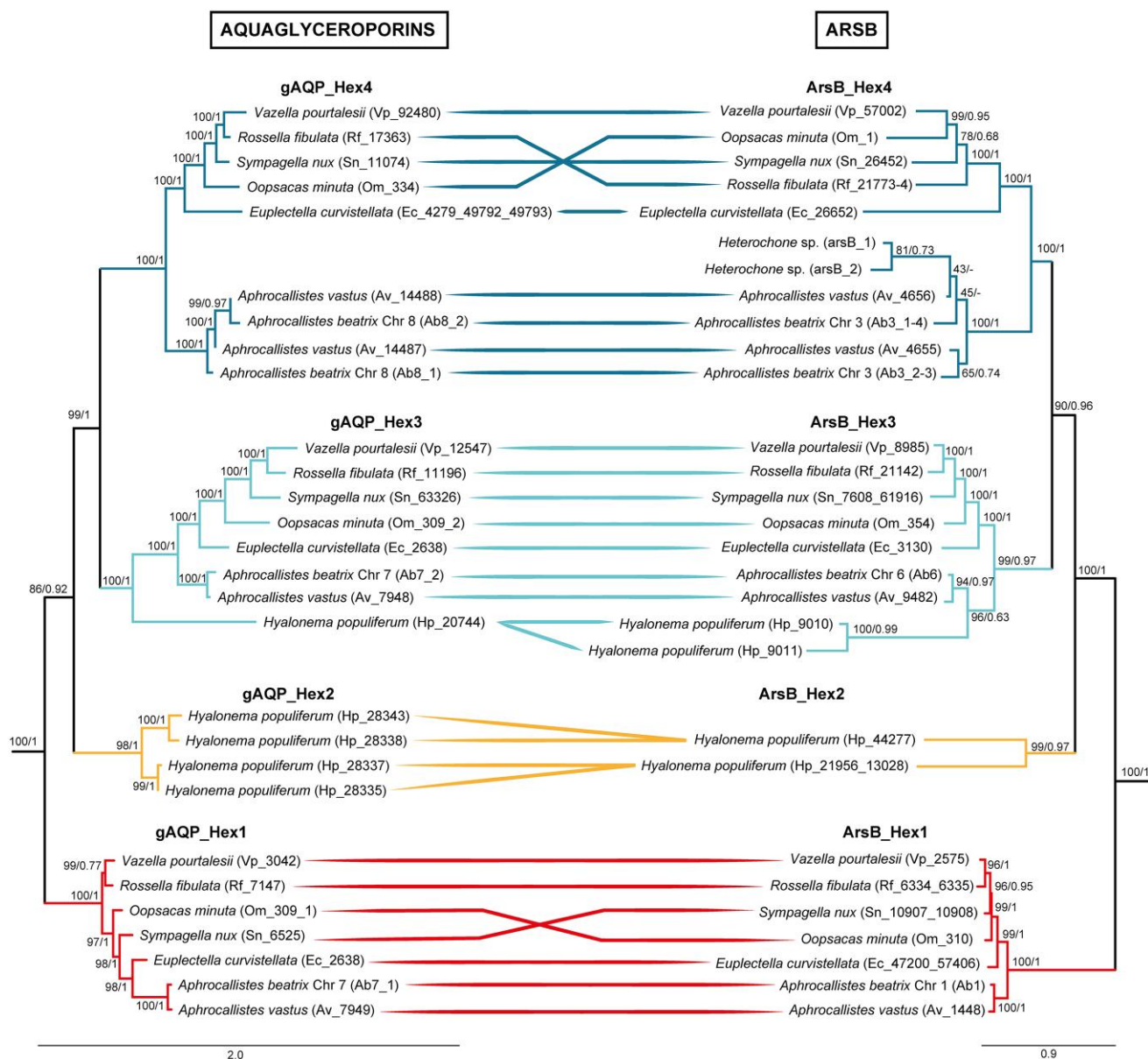


Figure 7 Coevolution of gAQP channels and ArsB pumps in hexactinellid sponges. Mirroring maximum likelihood phylogenetic trees illustrating the remarkable parallel evolution experienced by gAQP (left) and ArsB (right) sequences across the diversification of hexactinellid sponges. Lines connecting both trees indicate co-occurring genes within the same genome, suggesting potential coevolution. Bootstrap support values and Bayesian posterior probabilities are shown at the nodes (bs/pp). Branch colors represent different phylogenetic clusters. Scale bars indicate the number of amino acid substitutions per site. Note that ArsB sequences of *Heterochone* sp., which were included in the analyses as isolated sequences rather than as part of a transcriptome or genome, cannot be reflected in the coevolution of the gAQP proteins because such counterparts were not considered when constructing the data set (see Materials and methods). See [File S2](#) for sequences.

for this hypothesis also comes from several cases in the Heteroscleromorpha dataset where the number of *ArsB* genes matches that of *gAQP* genes ([Table S6](#)), such as in the poecilosclerid *C. crambe* or the tetractinellid *T. swinhoei*. In the genome of *C. crambe*, there are three *ArsB* genes and three *gAQP* genes available for functional pairing, but only one encodes ancestral Clade-2 *gAQP* channels, while the other two encode *gAQP*_Het_Chloroflexi channels derived from the gene acquired via HGT ([Table S6](#)). Likewise, the tetractinellid *T. swinhoei* possesses genes for four *ArsB* pumps that should be paired by four ancestral *gAQP* channels. However, the genome contains only

one ancestral *gAQP* channel and three *gAQP*_Chloroflexi channels acquired via HGT ([Table S6](#)). These examples support the hypothesis that *gAQP*_Het_Chloroflexi channels, acquired via HGT, began coupling with *ArsB* pumps for dSi transport, largely (but not completely) displacing ancestral Clade-2 *gAQP* channels from this role. The replacement disrupted the neat coevolutionary pattern between ancestral *gAQP* channels and *ArsB* pumps observed in Hexactinellida, a lineage that did not receive the *gAQP*_Het_Chloroflexi gene ([Figs. 7 and 8](#)). The evolutionary irruption of the HGT-acquired *gAQP* channel in Heteroscleromorpha Demospongiae but not in Hexactinellida

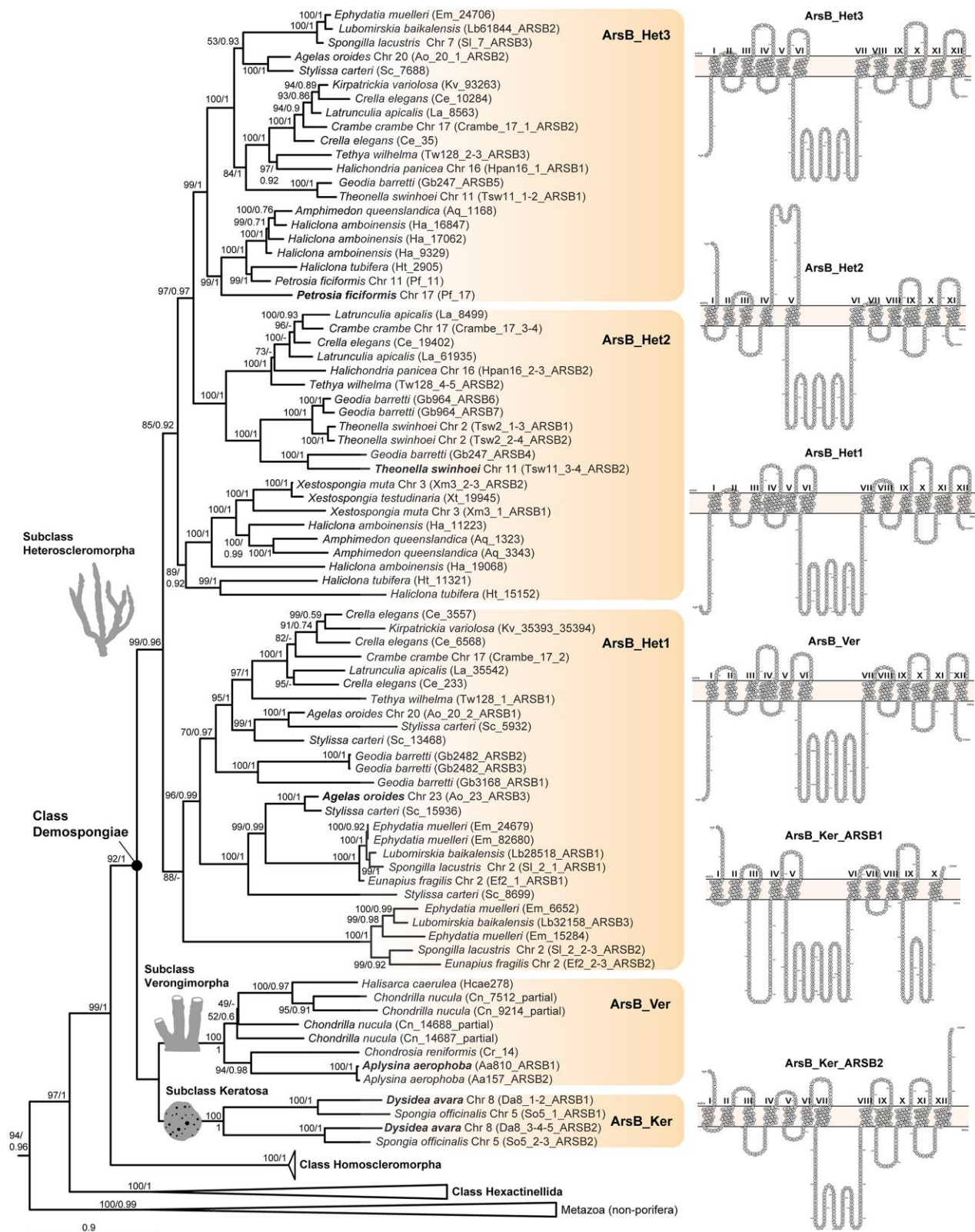


Figure 8 Phylogenetic relationships of demosponge ArsB transporters and secondary structure of clade representatives. The phylogeny was inferred with maximum likelihood via IQ-TREE software and showed identical topology when inferred with Bayesian methods. Numbers at nodes correspond to values of bootstrap support and posterior probability (bs/pp), respectively. Labels in brackets after the name of taxa are sequence codes in the alignment. Scale bar represents the number of amino acid substitutions per site. Secondary structure was inferred using Protter software for a representative gAQP member of each clade (species names in bold). Labels in Roman numerals in the secondary structure identify different transmembrane protein domains. Loops above and below the membrane correspond to extracellular and cytosolic regions, respectively. See [File S2](#) for sequences.

represents a so far unrecognized difference in the silicification process between these two sponge classes, the functional consequences of which remain to be elucidated. It cannot be discarded that the acquisition of gAQP_Chloroflexi channels enabled Heteroscleromorpha demosponges to better adapt to environments with low dSi, particularly following the expansion of diatoms in the post-Cretaceous oceans (Maliva et al. 1989; Siever 1991; Maldonado et al. 1999). This view is in line with observed distribution of these two sponge classes in the modern ocean. Hexactinellids do not normally live in low-dSi concentrations, inhabiting mostly dSi-rich deep waters ($>10 \mu\text{M}$ dSi) and polar environments, while demosponges are distributed ubiquitously through all dSi ranges of the ocean (Alvarez et al. 2017). The restricted habitat distribution of Hexactinellida could derive, at least in part, from a demonstrated poor performance of their Si uptake systems (ie ancestral gAQP channels) to initiate Si uptake under low-dSi concentrations (Maldonado et al. 2020), a subject which requires further investigation.

Yet, the putative competition established between ancestral and HGT-acquired gAQP channels for pairing with ArsB pumps appears to be a complex process, which shows diverse patterns over the phylogenetic tree (Fig. 8; Table S6). This includes some hard to explain unpaired numbers of antagonistic transporters, such as that in *Haliclona amboinensis*, where there are five ArsB pumps, but only two ancestral gAQPs and one HGT-acquired gAQP (genome data), or *Crella elegans* (transcriptome data), where 6 ArsB pumps are identified versus only one ancestral gAQP channel. This variety of patterns anticipates a complex diversification of the dSi transport pathway across Heteroscleromorpha, whose complete understanding will require further research.

Homoscleromorph ArsB transporters: genes lost but silicification regained

The clade corresponding to Homoscleromorpha sponges is represented by only two ArsB sequences belonging to the species *Corticium candelabrum* (Fig. 6b). One sequence occurs in chromosome 1 and the other in chromosome 2. No ArsB sequences were detected in the two other homosclerophorid species considered in the dataset, *Oscarella carmela* (transcriptome data) and *Oscarella lobularis* (chromosome-level genome data). Interestingly, while the genus *Corticium* (together with six other homoscleromorph genera) produces siliceous skeletons, the genus *Oscarella* (along with three other homoscleromorph genera) lacks siliceous skeletons. Our genomic explorations suggest that Homoscleromorpha sponges lack gAQP homologs employed for dSi transport in other sponges and that ArsB sequences are absent from the genome of *O. lobularis* (a non-silicifying species) but not from the genome of *C. candelabrum* (a silicifying species). Such a character distribution suggests that the gAQP precursors for dSi transport, inherited from early poriferans, were lost. This likely happened in the common ancestor shared with Calcarea (Fig. 1a) and before the emergence of silicification, at a time when the high ambient dSi concentration (close to saturation) caused important dSi diffusion into sponge cells through the ancestral gAQP channels, even if not yet specialized in dSi uptake. As no machinery for dSi polymerization had still evolved, the entering dSi was likely to cause homeostasis problems. In contrast to gAQP channels, ArsB pumps were lost specifically in non-

silicifying Homoscleromorpha sub-lineages. The key takeaway is that a system of paired gAQP-ArsB transporters for dSi, as seen in Hexactinellida and Heteroscleromorpha demosponges, was never functional in the Homoscleromorpha lineage, essentially because gAQP channels were lost before the emergence of silicification. Whether the retained ArsB pumps still contribute to dSi efflux remains unknown. However, their absence in non-silicifying species suggests that they may still play a role in dSi transport.

In this context, it remains unclear how 7 of the 11 Homoscleromorpha genera are able to produce silica skeletons, despite genomic analyses confirming the absence of ancestral Clade-2 gAQP channels for dSi transport and the lack of silicifying proteins known from Demospongiae (silicateins) or Hexactinellida (hexaxilin, glassin, perisilin). Therefore, the ability to silicify in these genera likely arose through the independent evolution of novel gene systems responsible for both dSi transport and polymerization, which have yet to be identified.

The fossil record of Homoscleromorpha, far younger than that of Heteroscleromorpha demosponges and Hexactinellida, also supports an independent origin of silicification. The oldest unambiguous fossil spicules for Homoscleromorpha date from the Upper Jurassic (161 to 145 mya) (Wiedenmayer 1994), and some less unambiguous skeletons from the Lower Carboniferous (360 to 299 mya) (Reid 2004) (Text S7). Molecular clocks estimate the origin of Homoscleromorpha in the Precambrian, that is, quite a few hundred million years before the oldest unambiguous Carboniferous-Jurassic fossil spicules for this sponge class. Some of these molecular approaches propose that Homoscleromorpha diverged from Demospongiae approximately 862 to 712 mya (Ma and Yang 2016). Others propose that Homoscleromorpha did not diverge from Demospongiae but rather from Calcarea around 850 to 700 mya (Dohrmann and Wörheide 2017). Irrespective of assignment of sister clade, both proposals support a Neoproterozoic origin for Homoscleromorpha. This implies that homoscleromorph sponges thrived in Paleozoic oceans without siliceous skeleton until some age between the Lower Carboniferous and the Upper Jurassic, that is, several hundred million years longer than Hexactinellida and Demospongiae, which developed biomineralized skeletons in the Lower Cambrian.

Structural comparison of dSi transporters

It is paradoxical that the gAQP-ArsB system for dSi transport in sponges is unrelated to that of other marine silicifiers but instead relies on distant homologs of Lsi1 channels and Lsi2 pumps, which are employed by land plants (Fig. 1b and c). The putative structural and functional similarities between sponge and plant dSi transporters remained largely unexplored and provide new insights into the evolution of silicon biomineralization.

Similarity between plant Lsi1 and human gAQP proteins 3 and 9 was assessed through sequence pairwise identity (PI%) and TM-score values. The latter ones provide a metric of the global topological similarity between two protein structures that ranges from 0 to 1, with 1 indicating a perfect match (Table S10). Protein comparisons returned quite low values (PI = 24.5 ± 2.9 , TM-score = 0.19 ± 0.02 , $N = 4$), despite all these types of aquaporin channels have been demonstrated to transport dSi (Ma et al. 2006;

Garneau et al. 2015). This comparison exemplifies that enormous divergence in sequence and structure does not necessarily mean loss of the ability to transport dSi in this protein family. In fact, average divergence between plant Lsi1 and sponge gAQP proteins is quite important too ($PI = 22.9 \pm 2.5$, $TM\text{-score} = 0.26 \pm 0.09$, $N = 7$). Within sponges, gAQP proteins have also diverged substantially through the diversification of Porifera, particularly in Heteroscleromorpha demosponges. Mean ($\pm$ SD) PI and $TM\text{-score}$ values are quite modest even within demosponge clades such as gAQP_Het ($PI = 49.4 \pm 6.3\%$, $TM\text{-score} = 0.25 \pm 0.08$) and gAQP_Het_Chloroflexi ($PI = 49.4 \pm 6.3\%$, $TM\text{-score} = 0.22 \pm 0.12$). In contrast, the value of these parameters is up to 3-fold higher within the hexactinellid clades (gAQP_Hex1: $TM\text{-score} = 0.66 \pm 0.14$, $PI = 85.9 \pm 4.6$; gAQP_Hex3: $TM\text{-score} = 0.76 \pm 0.05$, $PI = 73.6 \pm 8.9$; gAQP_Hex4: $TM\text{-score} = 0.67 \pm 0.27$, $PI = 78.5 \pm 10.8$). These figures reflect a much greater biological diversification in Demospongiae (8,063 species)—particularly in its subclass Heteroscleromorpha (7,243 species)—than in Hexactinellida (709 species). They also inform that the 3D structure has been much better preserved through the diversification of hexactinellid gAQP proteins (Fig. S3, Text S8).

The global comparison between plant Lsi2 and sponge ArsB pumps from all poriferan clades returns again low PI and $TM\text{-score}$ values ($PI = 27.5 \pm 2.7$, $TM\text{-score} = 0.19 \pm 0.04$, $N = 10$), testifying for significant sequence divergence between plants and sponges. By sponge clades, the maximum PI to Lsi2 was obtained versus the class Homoscleromorpha ($PI = 31.5\%$, $TM\text{-score} = 0.17$) and the minimum versus Keratosa demosponges ($PI = 23.7\%$, $TM\text{-score} = 0.16$; Table S11). Within Porifera, global sequence divergence among all sponge ArsB clades (mean $PI = 38.8 \pm 4.7\%$, $N = 45$) was slightly larger than among all sponge gAQP proteins (mean $PI = 44.4 \pm 12.5\%$, $N = 15$), irrespective of considering silicifying or non-silicifying clades. The highest $TM\text{-score}$ values for comparison of sponge ArsB proteins were consistently lower than 0.5, reflecting again an overall low structural similarity within Porifera.

Divergence has not only involved shifts in aa sequences, but also alterations in protein domains. Two-D models of gAQP proteins showed most Demospongiae and Hexactinellida sequences in Clade 2 to be typically characterized by six transmembrane domains, three extracellular domains, and four cytoplasmic domains, the latter including the N-terminal and C-terminal regions of the protein (Fig. 4, Table S12). This organization matches the standard for aquaporins (Agre 2004). However, there were two exceptions: (i) Hexactinellid proteins of subclade gAQP_Hex1 (except that of *Euplectella curvstellata*) differed by having seven transmembrane domains (TMs) instead of six, four extracellular domains instead of three, and a predicted extracellular N-terminus rather than a cytoplasmic one (Fig. 5); (ii) Heteroscleromorpha gAQP_Het_Chloroflexi proteins of Clade 1 exhibited significant nonsystematic variation in domain number, with transmembrane domains ranging from 4 to 8 across taxa, extracellular domains ranging from 2 to 4, and cytoplasmic domains ranging from 3 to 5 (Fig. 3, Table S12). This diversity in domain number reflects an important expansion and diversification of the gAQP_Het_Chloroflexi proteins that was coincidental with an extraordinary skeletal radiation in the subclass Heterosclerophora. The exact functional effects of domain variants of gAQP_Hex1 and gAQP_Het_Chloroflexi proteins remain unexplored.

The 2D structure of a majority of sponge ArsB transporters described in the present study consisted of 12 transmembrane domains, 6 extracellular domains, and 7 cytoplasmic domains. Some variations of that common pattern occurred, depending on ArsB clade/type (Table S13, Figs. 6 and 8). As also observed in plant Lsi2, the largest cytoplasmic domain—putatively responsible for the interaction with dSi—is located after the sixth TM of the protein. However, we discovered that the length of this cytoplasmic domain systematically changes across sponge clades. For example, within Hexactinellida, the average length of the largest cytoplasmic domain in ArsB_Hex3 is 187 aa, while in ArsB_Hex4 is only 38 aa (Table S13), testifying for notable protein divergence among different sponge clades across the three sponge classes and irrespective of producing or not a siliceous skeleton. Modeling of 3D structures indicated general agreement with the known structure of bacterial ArsB proteins (Fig. S3, Text S7).

Structural protein properties related to selectivity in Si transport were also examined, comparing well-studied plant species with several sponge species, which were chosen at random to represent the major sponge lineages.

Plant Lsi1 channels possess a transmembrane helical orientation different from other aquaporins and bacterial GlpFs (Wang et al. 2005), characterized by a widely opened, hydrophilic selectivity filter (SF) on the extracellular side. In rice (*Oryza sativa*), this SF is composed of six residues that create the narrowest portion of the channel (Thr65, Gly88, Thr181, Ser207, Gly215, Gly216, Arg222) and are critical to dSi selectivity (Saitoh et al. 2021). These aa are positioned distantly in the sequence but are brought close together upon protein folding to form part of the pore structure (Fig. 9a). The three glycines (Gly88, Gly215, Gly216) are arranged as the vertices of a hypothetical triangle, while the two threonines (Thr65 and Thr181) are positioned facing each other in a pseudo-symmetrical arrangement (Fig. 9a). These two Thr residues are responsible for strictly orienting silicic acid molecules when passing through the channel and represent a singularity of the Si-channel Lsi1, and their manipulation can modify the transport substrate specificity (Saitoh et al. 2021). On this basis, sponge gAQP sequences were examined for those seven amino acid residues. The only residue conserved in all sponge gAQP proteins was arginine (R222), with the presence/absence of the other residues varying, depending on the sponge gAQP clade (Table S14). On average, hexactinellid gAQP sequences exhibited the highest degree of conservation, with all four hexactinellid clades conserving at least three out of seven residues, and gAQP_Hex2 and gAQP_Hex3 clades conserving respectively six and five. In contrast, demosponge gAQP sequences in Clade 2 (gAQP_Het and gAQP_Ker) conserve only two amino acids (Gly 215 and Arg 222) and those in Clade 1 (gAQP_Het_Chloroflexi) just one (Arg 222). These features suggest that the dSi flux through hexactinellid gAQP channels is probably governed by Si selectivity mechanisms more similar to those operating in plants than demosponge gAQP channels. These differences in conservation of the Si selectivity filter between hexactinellid and demosponge gAQP channels are in line with the emerging view that Hexactinellida and Demospongiae may fractionate Si isotopes differently during transport (Maldonado and Hendry 2025).

More importantly, although the aa residues of the hydrophilic dSi selectivity filter of *O. sativa* Lsi1 channels (which are conserved through angiosperm species) are only moderately to poorly conserved in the studied sponge gAQP channels, the

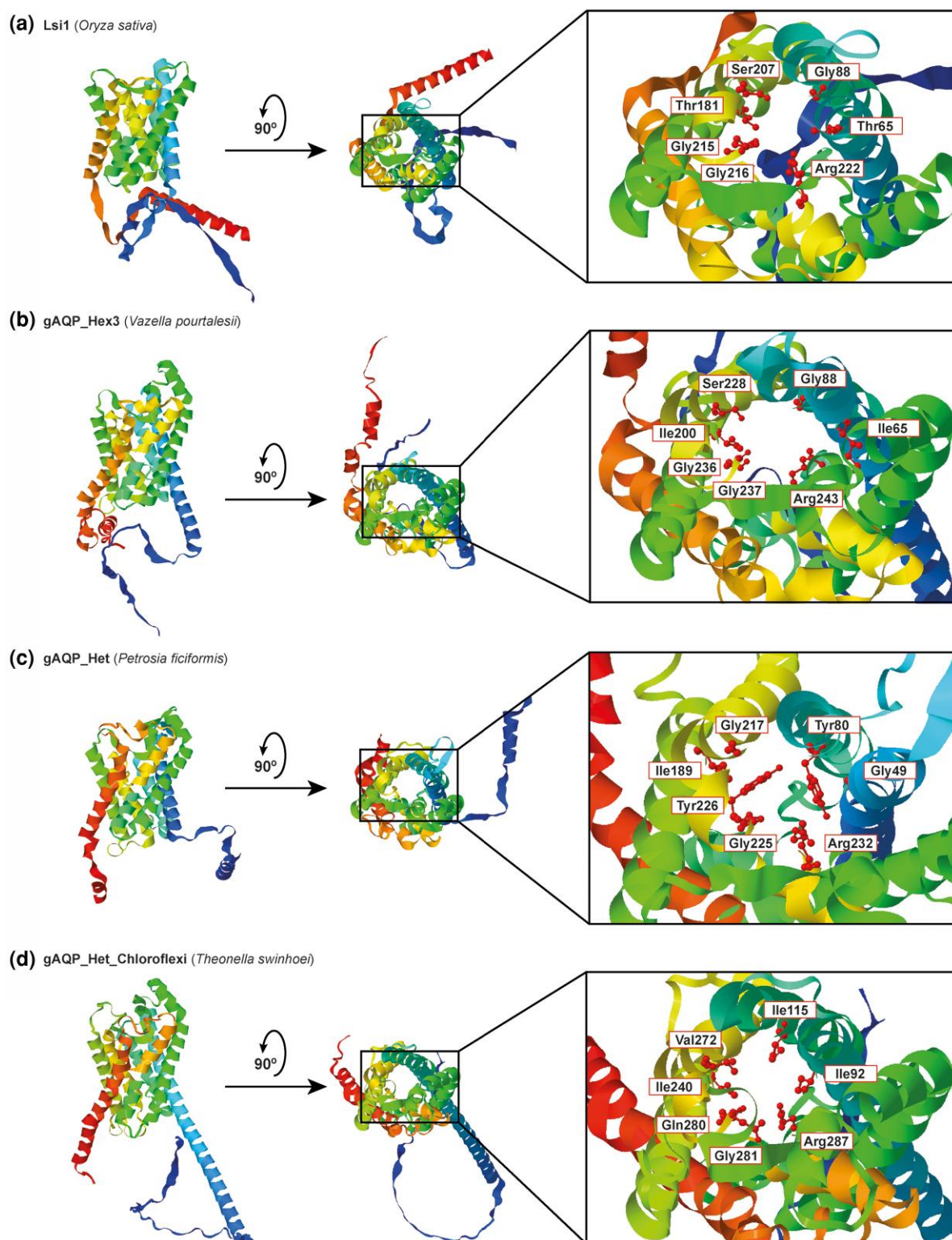


Figure 9 Structure of the silicon selectivity filter in gAQP channels. a) Structure of Lsi1 from *O. sativa*, a well-characterized silicon transporter, is shown in a side view (left), a top-down view after a 90° rotation (middle), and in a zoomed-in view of the pore and silicon selectivity filter (SF), as seen from the extracellular side (right). The inset highlights key amino acids involved in silicon selectivity (in red boxes). b to d) Structural models inferred for sponge aquaglyceroporins: b) gAQP_Hex3 from *V. pourtalesii*, c) gAQP_Het from *P. ficiformis*, and d) gAQP_Het_Chloroflexi from *T. swinhoei*. The left and middle panels follow the same visualization scheme as in a, while the right panels highlight the amino acids found at equivalent positions in these proteins. Essentially the hydrophilic aa residues of the plant SF have been replaced by hydrophobic residues, which is expected to reduce the transport of water during silicon transport. See [Table S14](#). Predicted template modeling scores (pTM) for the 3D models, which offer an integrated measure of how well AlphaFold multimer predicts the structure, are provided in [Table S15](#). PDB files for 3D models are provided in [Files S4 to S7](#).

patterns of substitutions are both intriguing and noteworthy when examined in the folded proteins (Fig. 9b to d). 3D modeling of a randomly selected ancestral gAQP channel for Hexactinellida, the gAQP_Hex3 protein of *V. pourtalesii* (Fig. 9b), revealed that its selectivity filter retains all three original glycine residues, as well as the serine and arginine found in the *O. sativa* Si selectivity filter. Remarkably, the two threonines have been replaced by a pair of isoleucine (highly hydrophobic, non-polar aa). The protein folding retains the spatial arrangement of these key residues as in *O. sativa*, despite sequence divergence, likely preserving functionality of the Si selectivity filter. This suggests that the functional role of this protein family is resilient to sequence variation. Replacing the two threonine residues with isoleucine is expected to transform the originally hydrophilic selectivity filter of *O. sativa* into a predominantly hydrophobic one in *V. pourtalesii*. The relatively hydrophilic selectivity filter of plant LSi1 channels allows permeation of silicic acid along with water (Saitoh et al. 2021; Saitoh and Suga 2022), which may facilitate subsequent transpirational dSi transport in the xylem (Fig. 1, Text S1). In contrast, following previous knowledge on GlpF and AQP selectivity filter (Fu et al. 2000; Tajkhorshid et al. 2002), it can be hypothesized that the markedly hydrophobic nature of sponge selectivity filter is expected to reduce water permeation during silicon transport, likely an adaptation to the marine environment lost in terrestrial plants.

Changes in the aa composition of the selectivity filter in demosponges (Fig. 9c and d), regarding both ancestral gAQPs and those acquired through horizontal gene transfer, are more pronounced than those in Hexactinellida, and also follow meaningful, nonrandom substitution patterns. In the gAQP_Het channel of *P. ficiformis* (Fig. 9c), three glycine residues still show a triangular arrangement, but their positions shifted to a chiral arrangement relative to that found in plants. Additionally, a pair of tyrosine residues (moderately hydrophobic, polar aa) is positioned opposite to each other, accompanied by an isoleucine (another hydrophobic residue) and an arginine, which, in this case, is the only conserved residue from the plant Si selectivity filter. Therefore, despite substantial sequence modifications, the spatial arrangement of the substituting residues suggests a functional selectivity filter, which, unlike in plants but similar to hexactinellid sponges, is largely hydrophobic (Fig. 9c).

In the gAQP_Het-Chloroflexi of *T. swinhoei*, the triangular arrangement is formed by isoleucine residues rather than glycines, although two glycines are still present, positioned opposite each other, alongside the conserved arginine. The serine residue has been replaced by valine, another hydrophobic aa. These changes result again in a highly hydrophobic selectivity filter (Fig. 9d).

Regarding the traits of the Si selectivity of sponge ArsB pumps, the insights obtained in this study are limited. We examined conservation of a GXQ motif located in the largest cytosolic loop of the protein, which has been linked to Si selectivity in plant Lsi2 transporters (Coskun et al. 2021). A multiplicity of similar GXQ motifs has also been suggested to be responsible for Si selectivity in diatom SITs (Knight et al. 2016). However, GXQ motifs were not present in any of the sponge ArsB proteins characterized in the present study. This outcome was not unexpected, since, while ArsBs and SITs both transport dSi, they are not homologous proteins (Marron et al. 2016).

Overall, it is remarkable that plants and sponges, despite their vast evolutionary divergence, have retained a homologous system for dSi uptake and subsequent internal transport. This

system appears to remain functional for dSi transport despite substantial divergence in both the sequence and secondary structure of its two antagonistic components. Such functional resilience may, in turn, have facilitated the physiological and ecological adaptability of siliceous sponges throughout the long evolutionary history of this metazoan group.

The emergence of sponge silicification

The ability to silicify relies on two key attributes: (1) a system to take up environmental dSi and transport it across cell membranes to the compartment where silicification occurs and (2) an “agent” able to promote polycondensation of the internalized dSi into skeletal biogenic silica, a function that, in all known sponge cases, is performed by silicifying proteins. In this study, we integrate the evolutionary insights on sponge dSi transporters obtained here with existing data on silicifying proteins and the fossil record, in order to reconstruct the key events underlying the emergence of silicification and the subsequent skeletal diversification in Porifera (Fig. 10).

Molecular clock estimates (Erwin et al. 2011; Ma and Yang 2016; Dohrmann and Wörheide 2017), together with evidence from silicifying proteins (Shimizu et al. 2024) and nonmineralized Proterozoic fossil remains (Turner 2021; Wang et al. 2024), including C31 sterols (Shawar et al. 2025), agree on placing the origin of Porifera and their divergence into classes in the Proterozoic (Cryogenian–Ediacaran, 900–635 mya; Fig. 10). Our results suggest that these Proterozoic sponges already possessed precursors of both modern gAQP channels (Figs. 3 to 5) and ArsB efflux pumps (Figs. 6 and 7), inherited from their common ancestor (Fig. 10). However, while the Proterozoic ancestors of sponges were endowed with the basic molecular elements of the gAQP-ArsB system, they still lacked the tools necessary to polymerize dSi into skeletal silica. This inference is supported by the fossil record, which shows that the first silica skeletons of Hexactinellida (around 528 mya; Chang et al. 2017) and Heteroscleromorpha Demospongiae (about 515 mya; Botting et al. 2015) appeared in the early Cambrian, and those of the verongimorph genus *Chondrilla* (Middle Devonian: 419 to 359 mya) and those of the class Homoscleromorpha much later (Upper Jurassic: 161 to 145 mya). In summary, the primordial molecular components of the gAQP-ArsB systems for dSi transport were present in the Porifera genome long before the protein machinery necessary to polycondense dSi into skeletal silica was operative.

For dSi polycondensation, hexactinellids have evolved at least three silicifying proteins (hexaxilin, glassin, and perisilin; Fig. 10), which are not phylogenetically related to each other (Shimizu et al. 2015, 2024). The current view is that hexaxilin forms the axial filament of all hexactinellid sponges and governs intracellular silicification, while perisilin and glassin function in the extracellular phase of silicification in different lineages. Unlike Hexactinellida, Heteroscleromorpha demosponges do not use several multiple silicifying proteins, but isoforms of a single enzymatic protein, silicatein (Shimizu et al. 1998a; Nishi et al. 2020), with different combinations of isoforms and their distinct expression patterns producing different spicule types (Müller et al. 2007b; Mohri et al. 2008). Silicatein evolved from a lysosomal digestive cathepsin-L enzyme, a process that turned the ancestral lysosome into a novel organelle, the silica deposition vesicle (Riesgo et al. 2015; Pozzolini et al.

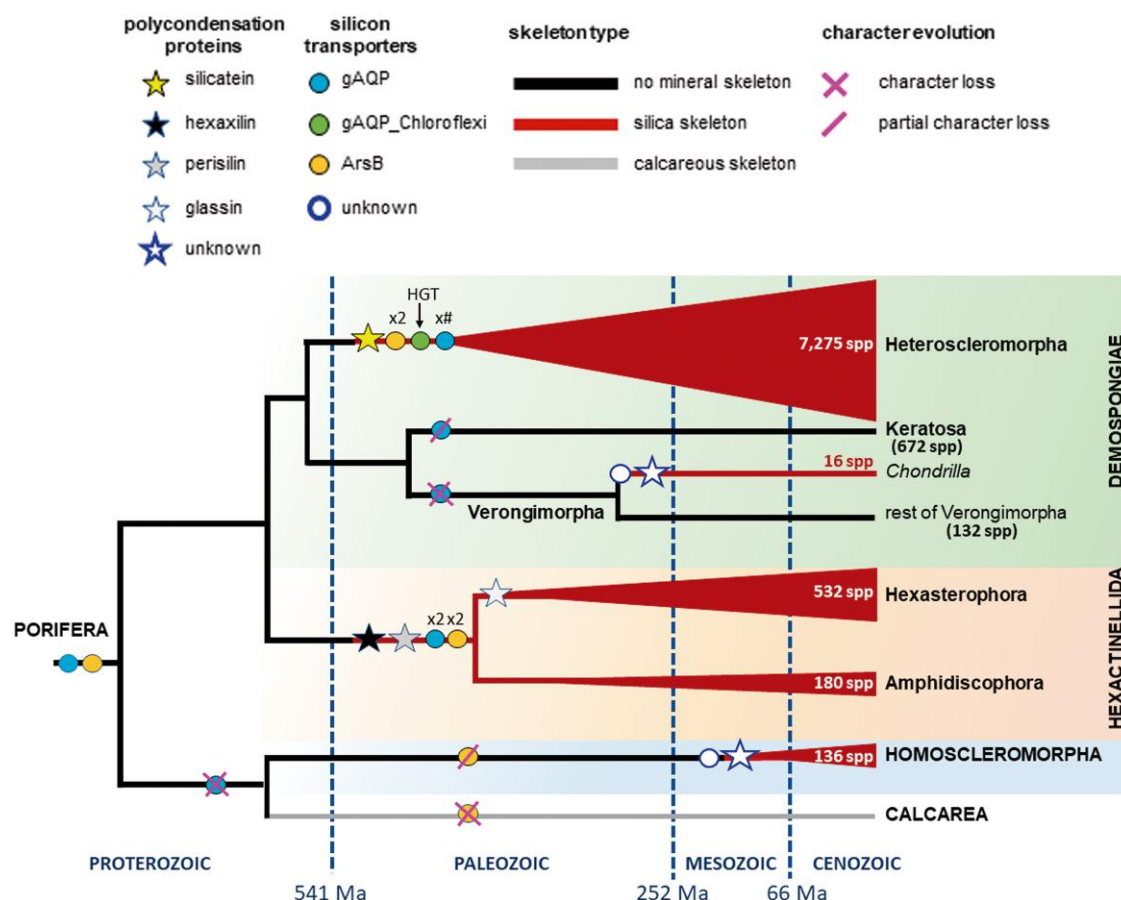


Figure 10 Hypothesis on the emergence of silicification in Porifera in relation to the evolution of dSi transporters and silicifying proteins through time (not to scale) and through lineage diversification and expansion (indicated by the number of extant species). The phylogeny begins with a poriferan ancestor lacking siliceous skeleton (black branches), but possessing ancestral transporters precursors of the gAQP and ArsB versions in modern sponges. Along the tree, it is summarized how the ability to produce siliceous skeletons (red branches) evolved independently at least four times and at different tempos during the diversification of the phylum, as a result of distinct acquisitions of silicifying proteins and varying patterns of diversification of dSi transporters. In Hexactinellida, silicification and skeletal evolution are linked to the acquisition of three exclusive silicifying proteins (hexaxilin, glassin, and perisilin), along with the parallel diversification of ancestral gAQP channels, and ArsB pumps through a remarkable process of coevolution, facilitated by systematic duplications (x2) in the ancestor of the class. In Demospongiae, only the subclass Heteroscleromorpha evolved silicifying proteins (silicateins). Here, duplications of ArsB efflux pumps (x2) were not mirrored by those of ancestral gAQP channels (x#), likely because gAQP_Het_Chloroflexi channels—acquired through horizontal gene transfer (HGT)—disrupted the coevolutionary trajectory. Note that these two exclusive novelties of Heteroscleromorpha demsponges (acquisition of the gAQP-Chloroflexi channel and silicatein enzyme) are coincidental with the most extensive skeletal diversification in the phylum. In the class Homoscleromorpha and the verongimorph genus *Chondrilla*, two small lineages with siliceous fossils known only from post-Cambrian periods, the emergence of silicification occurred through unidentified mechanisms different from those known from Hexactinellida and Demospongiae. In lineages that never evolved silicification or did so only after the Cambrian, the gAQP-ArsB system lost its gAQP component either partially or entirely (Text S9), likely to prevent dSi entry in cells that were unable to inactivate it by polymerization.

2022) (Text S3, Fig. S3). Contrary to previous suggestions (Müller et al. 2007a; Kozhemyako et al. 2010), genomic explorations have corroborated that silicateins are absent from the genomes of demsponges in the subclasses Keratosa and Verongimorpha, as well as from the class Hexactinellida, and are, therefore, an autapomorphy of Heteroscleromorpha demsponges (see Text S3, Fig. S3). The silicifying “agents” responsible for silica synthesis in the other two siliceous lineages of Porifera—ie the class Homoscleromorpha and the verongimorph demsponge genus *Chondrilla*—remain unidentified.

Regarding dSi transporters, our study reveals that each of the components of the ancestral gAQP-ArsB system underwent markedly different processes of expansion and diversification throughout the evolution of the phylum Porifera, depending on whether particular

lineages also acquired silicifying proteins (Fig. 10). In Hexactinellida, a gAQP-ArsB transport system was inherited from their ancestors and subsequently diversified through systematic duplications, showing a remarkable coevolution of its gAQPs and ArsB components. In the subclass Heteroscleromorpha of Demospongiae, the ancestral gAQP-ArsB system was modified (likely improved in terms of transport efficiency) through the acquisition of a bacterial GpIF channel for glycerol transport via lateral gene transfer, probably from a Chloroflexi symbiont. An important feature identified in these dSi transporters is that their Si selectivity filter has been modified through evolution to become markedly hydrophobic, compared to bacterial GpIF and plant Lsi1 transporters. This adaptation likely increases efficiency by minimizing water permeation during dSi transport. Since the HGT-acquired channel probably evolved to provide more efficient

dSi transport, it began to displace the ancestral gAQP from their pairing with the ArsB pumps in some species, resulting in a quite disorganized expansion of the gAQP-ArsB system during the diversification of Heteroscleromorpha demosponges (Fig. 7), notably different from the remarkable coevolution between the ancestral gAQPs channels and ArsB pumps seen in Hexactinellida (Figs. 8 and 10).

More importantly, the two autapomorphic innovations of Heteroscleromorpha demosponges (ie a HGT-acquired gAQP and silicatein) coincided with (and may have been responsible for) an extraordinary radiation and skeletal diversification in this subclass (Fig. 10), which currently comprises 7,275 spp. and represents about 74.4% of extant Porifera (9,781 spp.). The other two demosponge subclasses, which did not acquire either of these innovations, remain comparatively poorly diversified (Keratosa, 672 spp.; Verongimorpha, 148 spp.) and still lack a silica skeleton, except for the Verongimorpha genus *Chondrilla* (16 spp.).

It is also worth noting that some sponge lineages lost the functionality of the ancestral gAQP-ArsB system, mainly due to the loss of the gAQP component and, interestingly, this trait appears to follow a consistent pattern (Fig. 10): The sponge lineages that lost their gAQPs never developed the ability to silicify—as in the cases of the class Calcarea (837 spp.) and the demosponge subclasses Keratosa and Verongimorpha (collectively 820 spp.)—or developed it many million years after Hexactinellida and Heteroscleromorpha, as in the cases of Homoscleromorpha (136 spp.) and the Verongimorpha genus *Chondrilla* (16 spp.). Thus, the gAQP-ArsB transport system appears to have been a key driver of skeletal diversification and the evolutionary radiation of Porifera and particularly for the demosponge subclass Heteroscleromorpha.

The evolution of the gAQP-ArsB transport system in Demospongiae reveals additional, intriguing complexities: While the gAQP genes underwent extensive expansion and diversification in the subclass Heteroscleromorpha, many species in the subclass Keratosa and all species in Verongimorpha have lost these channels but still retain the ArsB genes (Fig. 10). The confirmed absence of gAQP proteins for dSi transport and silicateins for dSi polycondensation in the genome of the verongimorph genus *Chondrilla* suggests that this verongimorph demosponge genus acquired the ability to silicify by evolving entirely novel systems for both dSi transport and polycondensation, which remain to be identified (Fig. 10).

The situation is similar for the fourth silicifying lineage of Porifera (Fig. 10), Homoscleromorpha, although it arises from a different evolutionary process. While the silicifying proteins of Homoscleromorpha remain unidentified and thus cannot help to explain the atypical silicification long noticed in this lineage (Maldonado and Riesgo 2007), the present study of their dSi transporters provides some insight. Phylogenetic analyses and genome exploration have revealed that the common ancestor of Homoscleromorpha and Calcarea likely lost its ancestral gAQP channels (Fig. 10). However, the ArsB proteins are still retained in Homoscleromorpha species capable of silicifying (eg *C. candelabrum*), whereas they have been lost in non-silicifying species (eg *Oscarella* spp.). This distinct pattern of character distribution suggests that Homoscleromorpha sponges, as in the previous case of the genus *Chondrilla*, never possessed a functional gAQP-ArsB transport system for dSi, essentially because the gAQP component was lost prior to the emergence of silicification. Whether the ArsB pumps retained in these two silicifying lineages (ie *Chondrilla* and Homoscleromorpha) remain a functional component of their still undescribed dSi transport systems

or have been co-opted for other roles cannot be determined by our approach.

In summary, the ancestral gAQP-ArsB system for dSi transport was expanded in Hexactinellida through coevolution and in Heteroscleromorpha demosponges via horizontal gene transfer of a novel gAQP-Chloroflexi channel. In contrast, the gAQP-ArsB system was never functional in Homoscleromorpha and Calcarea and became nonfunctional in Verongimorpha demosponges and many sub-lineages of Keratosa demosponges. Except for Calcarea, which lost both ArsBs and gAQPs, the failure of the gAQP-ArsB transport system was recurrently due to the evolutionary loss of only its gAQP component (Fig. 10), a phenomenon circumscribed to lineages that either never evolved silicifying proteins or did so long after the Cambrian period. This pattern suggests that the gAQP channels, originally optimized for glycerol transport, allowed passive dSi leakage into sponge cells even before they became specialized for dSi transport. Precambrian and Cambrian oceans were characterized by very high concentrations of dSi (Maliva et al. 1989, 2005; Jurkowska and Świerczewska-Gładysz 2024), making uncontrolled passive diffusion of dSi into the sponge cells a potential threat to sponge homeostasis. In this scenario, sponge lineages that never evolved proteins capable of precipitating dSi (ie Keratosa, Verongimorpha, and Calcarea) or that acquired them long after the Cambrian (ie Homoscleromorpha, *Chondrilla*) lost gAQP channels, likely as a strategy to avoid or minimize unwanted dSi influx. In contrast, ArsB efflux pumps were retained in these non-silicifying groups, likely to remove trace amounts of dSi that still permeated (ie leakage) into sponge cells through conventional AQP channels nonspecialized for dSi but for glycerol, glucose, or other crucial solutes. This active removal (ie dSi efflux) is energetically costly, as it requires transporting dSi out of the cells against a concentration gradient, which was particularly steep in ancient oceans. In this scenario, only the nascent sponge lineages that soon evolved proteins capable of precipitating dSi into silica were able to mitigate the harmful effects of passive dSi influx. The resulting silica structures likely became advantageous because they provided skeletal functions (ie the emergence of skeletonization), which in turn conferred additional adaptive benefits to the early silicifying sponges, favoring a further specialization of the systems for dSi transport and polycondensation, which facilitated skeletal radiations.

This scenario therefore supports—following Shimizu et al. (2024)—that the emergence of silicification in Porifera represents an adaptation of early sponges to cope with the harmful effects of the very high dSi concentrations that characterized early oceans. This adaptation transformed a detrimental environmental challenge into a key innovation that initially enabled acquisition of a skeleton and subsequently facilitated lineage diversification. The evolutionary history of dSi transporters and the fossil record indicate that such adaptation arose independently on four separated occasions within Porifera.

Materials and methods

Data sources and general protocols

An updated database was compiled by selecting 40 publicly available genomes and transcriptomes for Porifera. The

database ensured a reasonable representation across taxonomic classes, subclasses, and orders, including genomic and transcriptomic data for 3 species of Homoscleromorpha, 27 species of Demospongiae, 8 species of Hexactinellida, and 2 species of Calcarea. Details of the sources for these genomes and transcriptomes are provided in Table S1. Hereafter, this sponge database will be referred to as the “local database.”

Putative dSi transporters were identified in the sponge database using ncbi-blast-2.12.0+ software, with the tblastn algorithm and the parameters e-value 0.05, matrix BLOSUM62, gap-open cost 11, and gap-extend cost 1. The amino acid (aa) sequence of aquaglyceroporin and ArsB proteins found to be upregulated in individuals of *V. pourtalesii* exposed to high dSi concentrations during experiments in Maldonado et al. (2020) were used as queries. These queries respectively corresponded to the complete sequences of aquaglyceroporin 3/9 protein (TRINITY_DN17190_c0_g1_i1) and to a long—but incomplete—sequence of ArsB1 protein (TRINITY_DN57791_c0_g1_i1), as referenced in the newest assembled transcriptome of *V. pourtalesii* by Shimizu et al. (2024) (Table S1). In these searches, only hits with a bitscore higher than 50 were retained, as this value has been shown to represent a suitable threshold to infer homology (Pearson 2013).

To analyze the retrieved sequences of sponge dSi transporters into a broad evolutionary context, two independent molecular datasets were built, one for aquaglyceroporins and the other for ArsB sequences. The aquaglyceroporin dataset included, in addition to the sponge data, sequences of other metazoan aquaglyceroporins 3, 7, and 9, plant NIP-like proteins, and both plant and metazoan sequences of classical water-selective aquaporins (1, 2, 4, 5, PIP/TIP, MIP) and AQP8-type aquaporins. We also included sequences of metazoan unorthodox aquaporins 11 and 12 plus bacterial and archaeal AqpZ and GlpF sequences. Classical water-selective aquaporins and unorthodox aquaporins were used as outgroup. The ArsB dataset comprised representatives of bacteria, diatoms, plants, choanoflagellates, and metazoans, along with the sponge data, which included two isolated sequences of the hexactinellid *Heterochone* sp., for which no genome/transcriptome of reference exists (Maldonado et al. 2020). Pink-eyed dilution (PED) proteins were used as outgroup.

Additionally, we conducted a blast search against NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and EukProt v3 databases (<https://evocellbio.com/eukprot/>) to complete the sponge datasets with non-sponge groups that were not represented or underrepresented in Maldonado et al. (2020). The blast search in NCBI was conducted using the program version BLASTP 2.15.0+ against all nonredundant GenBank CDS translations, PDB, Swiss-Prot, PIR, and PRF databases. The blast search at EukProt was performed using the Sequence Server 2.0.0 with the BLASTP 2.13.0+ program version against the complete database, which included 993 eukaryote genomes with representatives of all the major eukaryotic supergroups. In both searches, we used the same query sequences and blast parameters as previously detailed for the local blast against the constructed poriferan database.

Aquaglyceroporin dataset

Blast searches of aquaglyceroporin 3/9 (gAQP3/9) from *V. pourtalesii* against our local Porifera database returned a total of 170 hits with bitscore higher than 50 (Table S2). Six of these hits were discarded after corroborating their assignment to classical

water-selective aquaporins, while 112 corresponded to species in the class Demospongiae and 46 to Hexactinellida. Surprisingly, none was found in the class Homoscleromorpha. There were also six sequences in Calcarea, which the phylogenetic analyses subsequently positioned out of the clade of sponge gAQP channels involved in dSi transport.

Notably, many hits of each individual were consecutively distributed in the same subject sequence, indicating either the putative existence of introns (in the case of genomes) or highly variable regions within those sequences, which the blast algorithm was not able to retrieve. To avoid overestimation of the diversity of aquaglyceroporin proteins in these individuals, we curated these sequences manually, looking for the open reading frames (ORFs) and putative intronic regions using the software Geneious Prime 2022.0.1 (<https://www.geneious.com>) and Miniprot (Li 2023). When ORFs and intronic regions were reliably identified, we retrieved the entire amino acid sequence of each individual instead of the partial hits.

The blast search of gAQP3/9 of *V. pourtalesii* against the NCBI database retrieved a total of 506 putative homologous sequences (last accessed 09/02/2024) (Table S3). Among these hits, 503 belonged to aquaglyceroporins AQP3, AQP7, AQP9, and AQP10 of metazoan species, while 3 of them were synthetic human vectors. Out of all these non-sponge metazoan hits, we included in our dataset only the two sequences of each aquaglyceroporin type with the highest bitscore. Finally, the blast search of gAQP3/9 of *V. pourtalesii* against the EukProt database retrieved a total of 502 hits (last accessed 09/02/2024) (Table S4). Among these hits, 266 corresponded to metazoan species and 236 to different lineages from the major eukaryotic supergroups (SAR, Excavata, non-plant Archaeplastida, Amoebozoa, and non-metazoan opisthokonts). For practical reasons, we incorporated into the dataset only a reduced but informative representation, consisting of 22 sequences (with the highest bitscore) belonging to the major non-metazoan eukaryotic supergroups. Thus, the final aquaglyceroporin dataset comprised a total of 201 sequences (File S1). Yet, we conducted additional searches in the case of Calcarea aquaglyceroporins, because a partial sequence of the species *L. complicata* was found to be similar to aquaglyceroporins of the siliceous sponges (Text S2). In order to shed light on such unexpected result, we searched three more recently published genomes within this class (viz. *Clathrina lutea*, *Nicola tetela*, and *Sycon ciliatum*) using the gAQP 3/9 of *V. pourtalesii* as a query. This blast search retrieved only partial sequences of less than 100 aa (Table S16), indicating that Calcarea aquaglyceroporins have diverged so much from the other sponge aquaglyceroporins that the blast algorithm is not able to retrieve complete sequences. Thus, the similarity of the partial gAQP sequence of *L. complicata* to siliceous sponge aquaglyceroporins might be an artifact due to sequence incompleteness.

ArsB dataset

Blast searches of ArsB1 from *V. pourtalesii* against our local Porifera database resulted in a total of 209 hits with a higher bitscore than 50 (Table S7). Among these hits, 144 were found in Demospongiae, 59 in Hexactinellida, 6 in Homoscleromorpha, and none in Calcarea. As in the aquaporin dataset, we curated the hits of each individual found in the same subject sequence

by looking for the ORFs and putative intronic regions using Geneious Prime and Miniprot software.

The blast search of *V. pourtalesii* ArsB1 against the NCBI database retrieved a total of 253 putative homologs, all within Eukaryota (last accessed 02/02/2024) (Table S8). Twenty-one of these sequences belonged to metazoan species, including four sponges, two mollusks and one brachiopod, while the rest of 232 sequences belonged to plants. The blast search against EukProt retrieved a total of 734 hits (last accessed 05/02/2024) (Table S9). Thirty of them corresponded to metazoan species, 224 to plants, and the remaining 480 to different lineages from the major eukaryotic supergroups (SAR, Excavata, non-plant Archaeplastida, Amoebozoa, and non-metazoan opisthokonts). For practical reasons, we selected a reduced representation from these blast hits for the ArsB dataset, which consisted of 19 sequences from all main eukaryotic supergroups that were not represented in the molecular dataset from Maldonado et al. (2020) (selecting the hits with the highest bitscore). Consequently, the final ArsB dataset comprised a total of 161 aa sequences (File S2).

Phylogenetic inference

The aa sequences of the gAQP and ArsB datasets were independently aligned using the MAFFT (version 7) online software with the E-INS-I iterative strategy (Katoh et al. 2019). Phylogenetic trees were estimated using both maximum likelihood and Bayesian inference methods, implemented in the programs IQ-TREE 1.6.0 (Nguyen et al. 2015) and MrBayes 3.2 (Ronquist et al. 2012), respectively. In IQ-TREE, the phylogenetic inference was performed using 5,000 replicates of ultrafast bootstrap (Hoang et al. 2018) and enforcing the ModelFinder Plus option, which searches and applies the best-fitting evolutionary model to the data (Kalyaanamoorthy et al. 2017). The best-fitting model of the ArsB alignment according to the Akaike Information Criterion (AIC) was the Le-Gascuel plus a ten-category FreeRate model for heterogeneity across sites (LG + R10). For the aquaglyceroporin alignment, the best-fitting model was also the Le-Gascuel, but with empirical amino acid frequencies and an eight-category FreeRate model for heterogeneity across sites (LG + F + R8). Phylogenies estimated with MrBayes 3.2 were inferred with two runs of 2,000,000 generations, four chains, sampling every 200 generations, and applying the default burn-in of 25%. For each alignment, the best-fitting evolutionary model from the ones available in MrBayes 3.2 was selected using ModelFinder (implemented in IQ-TREE 1.6.0). For the ArsB, the best-fitting model was the Whelan and Goldman plus a four-category discrete gamma distribution (WAG + G4), while for the aquaporins, it was also the Whelan and Goldman plus a four-category discrete gamma distribution with invariable sites (WAG + I + G4). For the Bayesian inference analyses, the convergence of the two runs was assessed by examining that the standard deviation of split frequencies was below 0.05. The trees obtained were visualized with FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>) and enhanced as final figures using Adobe Illustrator 2023 (version 27.1.1).

Silicatein occurrence in nonsiliceous demosponges

There are two reports on the presence of silicatein in the demosponge subclass Keratosa (Kozhemyako et al. 2010; Riesgo et al. 2015), which are controversial for two reasons: (i) They complicate

the mapping of the presence/absence of silicatein on the poriferan phylogeny; (ii) they are based on relatively short, incomplete sequences that favor artifactual results. We leveraged newly available high-quality genomes of several representatives of the subclasses Keratosa and Verongimorpha to further investigate the presence of silicatein enzymes in their nonskeletonized members, as well as in two species of *Chondrilla*, which produce a silica skeleton. To do so, we performed a blast search using the silicatein alpha sequence of *Tethya aurantium* as a query (GenBank Acc. Num. AAC23951.1), against 12 genomes of Keratosa and Verongimorpha (Table S17). For Keratosa, we included the species *Dysidea avara*, *Ircinia fasciculata*, *Phyllospongia foliascens*, *Rhopaloeides odorabile*, *Spongia lamella*, and *Spongia officinalis*. For Verongimorpha, we used the species *Aplysina aerophoba*, *Chondrilla caribensis*, *C. nucula*, *Chondrosia reniformis*, *Halisarca caerulea*, and *Ianthella basta*. Since silicateins are very similar to cathepsins in sequence, we compiled a comprehensive representation of sponge silicatein and cathepsin sequences to correctly identify the hits retrieved by the blast in Keratosa and Verongimorpha (File S3) through subsequent phylogenetic analysis. A total of 124 sequences were aligned using the software MAFFT online, and a maximum likelihood phylogeny was inferred using the program IQ-TREE 1.6.0. The phylogenetic inference was run setting 5,000 replicates of ultrafast bootstrap (Hoang et al. 2018) and enforcing the ModelFinder Plus option, which searches and applies the best-fitting evolutionary model to the data (Kalyaanamoorthy et al. 2017), which was the Whelan and Goldman plus a five-category model for heterogeneity across sites (WAG + R5) (Fig. S4).

Secondary and tertiary structure

The secondary structure of aquaglyceroporins and ArsB transporters identified in Porifera was analyzed using two different web-based tools: Phobius software (Käll et al. 2007), accessible at <https://phobius.sbc.su.se/> and Protter version 1.0 (Omasits et al. 2014), accessible at <https://wlab.ethz.ch/protter/start/>. Phobius was run under the normal prediction approach. Both programs are able to infer the extracellular, cytoplasmic, and transmembrane domains of the proteins based on the amino acid characteristics. Phobius results for secondary structures were exported in text format, while Protter results were exported as snake plots in png format.

The tertiary structure of the identified sponge aquaglyceroporins and ArsB transporters was inferred using AlphaFold3 (Abramson et al. 2024), implemented in the AlphaFold Server (<https://alphafoldserver.com/welcome>). The top-ranked 3D protein structure of each prediction was downloaded in PDB format and examined using the software Geneious Prime. The reliability of the 3D predictions was evaluated using predicted template modeling (pTM) metrics. The pTM score, which ranges from 0 to 1, measures the prediction accuracy of the entire structure. Values between 0.5 and 0.7 indicate some uncertainty between domains, while values higher than 0.7 indicate a trustworthy global structure (good domain positioning).

2D and 3D protein similarity

Pairwise identity (PI, %) at the amino acid level was calculated using the software Geneious Prime. Topological similarity of protein tertiary structure was assessed using TM-score software

(Zhang and Skolnick 2004). The TM-score ranges from 0 to 1, where 1 indicates a perfect match between two structures, values between 0.5 and 1 suggest that proteins share the same fold, and values below 0.17 indicate random structural similarity. For aquaglyceroporins, PI and TM-score values were calculated among the different monophyletic gAQPs groups identified in Porifera, as well as between these groups and the four human aquaglyceroporins known to transport dSi in various tissues (GenBank Accession Numbers Q92482.2, O14520.1, KAI4058012.1, NP_536354.2), along with the plant Lsi1 silicon transporter from *O. sativa* (GenBank Accession Number Q6Z2T3.1). For ArsB transporters, PI and TM-score values were calculated across the different ArsB groups found in Porifera, also between them and the plant Lsi2 silicon transporter from *O. sativa* (GenBank Accession number ADH94038.1).

Identification of SA specificity sites

We analyzed the sponge gAQP and ArsB sequences identified in this study for conservation of aa residues that have been demonstrated or postulated to confer Si selectivity during transport in homologous proteins, as well as in nonhomologous proteins with the same function on dSi transport (eg SITs). In the case of aquaglyceroporin sequences, we searched for the specific aa residues known to act as a selectivity filter for dSi in the Lsi1 transporter of plants (Saitoh et al. 2021; Saitoh and Suga 2022). These amino acids correspond to Threonine65, Glycine88, Threonine181, Serine207, Glycine215, Glycine216, and Arginine222, in the reference Lsi1 sequence of *O. sativa* (GenBank Accession number Q6Z2T3.1). To do so, we aligned the Lsi1 reference sequence of *O. sativa* with the different sponge gAQP types identified in the present study using the software MAFFT v. 7 online, with the E-INS-I iterative strategy. These particular amino acids (or the amino acids found at the same position) were also identified in the 3D structure of the gAQP proteins using the software Geneious.

For the ArsB transporters, we searched for conservation of GXQ motifs, which had been suggested to be responsible for Si selectivity in diatom SIT transporters (Knight et al. 2016), also discussed as part of the Lsi2 transporters in plants (Coskun et al. 2021).

Statistics and reproducibility

The study does not include experiments. Data are sequences publicly available, as indicated in the “Materials and methods” and “Data availability” sections. When required, data were filtered under objective, quantitative criteria, as it is explained in the respective methodological sections. Unexplained, subjective data exclusion was not conducted. Significance values used for statistical tests and processes are explained in Materials and methods, and statistics are presented in text or figures in all cases. Data were processed using NCBI-blast 2.12.0, MAFFT, IQ-TREE 1.6.0, MrBayes 3.2, Phobius, Protter, AlphaFold3, and Geneious Prime.

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Author contributions

L.L. and M.M. contributed equally to study design, data analysis, interpretation of results, and writing of the manuscript.

Supplementary material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Conflicts of interest

None declared.

Data availability

Links for all poriferan genomes and transcriptomes used in the present study are provided in Table S1 associated with the manuscript. All characterized amino acid sequences are available as supplementary data files associated with the manuscript and have also been deposited at the Figshare data repository (<https://doi.org/10.6084/m9.figshare.28760315>). All quantitative and qualitative raw data supporting the findings are given in Tables S1 to S17 and Figs. S1 to S4. No specific code was written or developed for this study.

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